PHYSIOLOGICAL ZOÖLOGY

Volume XXIII APRIL 1950 Number 2

STUDIES ON THE QUESTION OF CHEMOAUTOTROPHY IN CHILOMONAS PARAMECIUM[1]

WILLIAM B. COSGROVE[2]
University College, New York University

ALTHOUGH the great majority of nonphotosynthetic organisms use organic compounds as carbon and energy sources, some bacteria are known to utilize inorganic oxidations for energy, and carbon dioxide as their only carbon source (see the review of van Niel, 1943). Until the report of Mast and Pace (1933), there was no evidence that such chemoautotrophic potentialities occur among the Protozoa; these authors reported that *Chilomonas paramecium* grew "in a sterile solution containing $MgSO_4$, K_2HPO_4, $NaC_2H_3O_2$ and NH_4Cl and in this solution with the acetate omitted, provided silicon is added and the CO_2 concentration of the air increased." Qualitative tests purported to show the appearance of nitrate during the growth of the chilomonads in the inorganic solution. Mast and Pace employed an isolation-culture technique and expressed their results in number of divisions per day. Hall and Loefer (1936), using a different strain of this flagellate and different media, were unable to maintain mass cultures in purely inorganic solutions. Burrows (1938) obtained populations of about 5000 chilomonads per cubic centimeter in Mast and Pace's medium fortified with calcium chloride, in volumes of 50–60 cc. in 125-ml. Erlenmeyer flasks plugged with cotton. The presence of sodium acetate did not increase the maximum population. However, he could not obtain positive nitrate tests and did not regard the observed increase in nitrite as significant.

Hutchens (1940) found that supplementing an acetate medium with $FeCl_3$ and thiamin resulted in populations of about 200,000 per milliliter. Since Mast and Pace's medium was deficient in these compounds, they, rather than organic materials, were probably the limiting factors in Burrows' cultures, explaining the failure of acetate to increase growth.

In any investigation of chemoautotrophy, rich and vigorous mass cultures are essential in attempting to demonstrate the energy-yielding reaction. Moreover, when only low maximum populations are obtainable, it is possible that small amounts of organic materials, undetected in the culture medium, are supporting growth. If it can be shown that an exergonic inorganic reaction catalyzed by the organisms supplies more

[1] A dissertation submitted to the Graduate School of Arts and Science of New York University in partial fulfilment of the requirements of the degree of Doctor of Philosophy. The author takes pleasure in acknowledging the generous advice, encouragement, and assistance of Professor R. P. Hall.

[2] Present address: Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

than enough energy to account for the organic carbon present in the organisms after growth, then it may be inferred that chemoautotrophy actually occurs, even though technical limitations prevent demonstration of a net increase in organic carbon. Conversely, if no such reaction can be demonstrated, then the possibility still remains that traces of organic materials are supporting the observed growth.

Because the original report of Mast and Pace does not fulfil these criteria and because of the conflicting results of other workers on this problem, the whole question of chemoautotrophy in *C. paramecium* has been reinvestigated.

MATERIALS AND METHODS

The strain of *C. paramecium* used was isolated in bacteria-free cultures by Pringsheim[3] and has been maintained for a period of years in this laboratory on peptone media. As a preliminary to these experiments, the organism was carried through ten serial transfers in Medium II plus 250 mg. per cent sodium acetate trihydrate and 0.001 mg. per cent thiamin hydrochloride, and stock cultures have since been maintained on that medium.

Pyrex glassware has been used throughout, except for the transfer pipettes and the vials covering the small flasks. Before use, all glassware was washed in hot Alconox solution, rinsed, and immersed overnight in strong chromic-sulphuric cleaning solution. It was then rinsed in six changes of hot distilled water, followed by four changes of hot Pyrex-redistilled water, and then steamed for 15 minutes. Both the results of Schoenborn (1949) with other flagellates and the very large populations ($0.2–1.2 \times 10^6$ per milliliter) in acetate or ethanol media in identically treated flasks appear to rule out any deleterious dichromate effect.

Pipettes and other dry glassware were sterilized by dry heat at 165°–70° C. for 3 hours; all solutions were sterilized at 15 pounds pressure for 20 minutes in an autoclave steamed out with flowing steam for 30 minutes before use.

The two inorganic media used, modified from those of Schoenborn (1946) and Burrows (1938), were of the following composition: Medium I (Schoenborn)—$(NH_4)_2HPO_4$, 1.00; $MgSO_4 \cdot 7H_2O$, 0.20; KH_2PO_4, 0.20; KCl, 0.20; $FeCl_3 \cdot 6H_2O$, 0.0025; $CaCl_2 \cdot 2H_2O$, 0.0013; $Na_2SiO_3 \cdot 9H_2O$, 0.2274 gm.; H_2O, 1.0 liter. Medium II (Burrows)—NH_4Cl, 0.4986; $MgSO_4 \cdot 7H_2O$, 0.0986; $K_2HPO_4 \cdot 3H_2O$, 0.2739; $CaCl_2 \cdot 2H_2O$, 0.0013; $FeCl_3 \cdot 6H_2O$, 0.0025; $Na_2SiO_3 \cdot 9H_2O$, 0.2274 gm.; H_2O, 1.0 liter. All the salts were reagent-grade products of Eimer and Amend or Mallinckrodt; the water was redistilled from an all-Pyrex still. Medium I was adjusted to pH 7.0 before autoclaving; after equilibration with the air-CO_2 mixture, the pH was 6.0–6.1. Medium II either was left unadjusted, giving an equilibration pH of 5.6–5.7 or was adjusted to pH 7.0 (equilibration pH 5.2–5.3). No difference in growth rate or maximum population between the two media or among the three pH values was detectable, provided that Medium II, when unadjusted, was equilibrated with the air-CO_2 mixture before inoculation.

For certain experiments the media were supplemented with thiamin hydrochloride (0.01 mg. per liter) or a vitamin supplement derived from the results of Pace (1947): nicotinic acid, 1.0; pyridoxine HCl, 1.0; thiamin HCl, 10.0; riboflavin, 1.0 mg.; 0.1 N HCl, 100 ml.

[3] Mast and Pace's original strain could not be obtained. However, Mast and Pace (1936), after studying strains isolated from various localities of the eastern United States, concluded that it was "not probable that there are different strains of *Chilomonas* in reference to ability to obtain nitrogen and carbon from simple compounds."

In both cases, the stock vitamin solution was autoclaved separately and was added aseptically to the sterile medium in the proportion of 0.1 ml. per liter of medium.

In preparing the media, all ingredients were weighed out separately into glass weighing bottles, with glass spatulas. They were then added to the required amount of water, and the bottles were rinsed with the solution; pH adjustments were made by the addition of N HCl. The medium was then dispensed from a burette into culture flasks in 9-cc. or 45-cc. amounts. During preparation of the medium, great care was exercised to prevent chance contamination with traces of organic materials.

Flasks were plugged with Johnson and Johnson nonabsorbent cotton or covered with inverted beakers or vials. The 25-ml. Erlenmeyer flasks were covered with small caps made by cutting off the end of a large test tube, fire-polishing, and then indenting the margin so that the inverted vial was held away from the wall of the flask, permitting ready diffusion of gases. Large flasks were covered with Pyrex beakers of appropriate size.

All populations were determined by the method of Hall, Johnson, and Loefer (1935) except for the very large populations in organic, vitamin-supplemented media, which were counted by hemocytometer. Initial populations were computed from the population of the stock culture except in the growth-curve studies where, immediately after inoculation, an aliquot was removed and the population counted. During counting, the organisms were observed for gross morphological abnormalities, and the frequency of dividing forms was noted.

Cultures were incubated at 23° C.; the inoculated flasks were placed in anaerobe jars or bell jars of known volume, and CO_2 from a Kipp generator or a cylinder of compressed gas was admitted slowly at the bottom, while the displaced air was collected and measured. Enough CO_2 was added to give an air-CO_2 ratio by volume of 5/1. This ratio was regarded by Mast and Pace as the most favorable.

Organic carbon determinations were made by the wet combustion method of Van Slyke and Folch (1940). Nitrite was determined by Shinn's (1941) method, slightly modified to use smaller volumes and give greater sensitivity. Nitrate was tested for, qualitatively and quantitatively, by the diphenylbenzidine method of Snell and Snell (1936), as used by Hutchens, Podolsky, and Morales (1948).

Frequent tests for bacteriological sterility were made, using both organic and inorganic (Winogradsky) media; data from the rare contaminated cultures were discarded.

EXPERIMENTAL RESULTS

SERIAL TRANSFER STUDIES

The serial cultivation of an organism on a new medium both dilutes out the constituents of the old medium and provides opportunity for adaptation or selection to occur. Attempts to establish *Chilomonas* on Media I and II either without added silicate or with K_2SiO_3 were unsuccessful. When sodium silicate was used, no difficulty was experienced in establishing four separate series on apparently inorganic media. Table 1 presents the results of three of these series. The populations are low and variable. Variations in the period of incubation between 96 and 240 hours have no real effect on the maximum population, except that very small inocula require longer to reach the maximum. Death of the organisms, apparently from starvation, occurs after 5–7 days. There is no correlation between the age of the medium and the population obtained. Certain

TABLE 1*

SERIAL TRANSFERS IN INORGANIC MEDIA

Series	Medium	Transfer No.	Hours Incubation	Organisms per Ml. ±S.E.	Population Growth
1.....	I	1	72	2,727±250	.....
		2	240	2,227±226	8.2
		3	76	759± 24	3.4
		4	90	1,218± 35	16.0
		5	106	1,488± 59	12.2
		6	135	1,287± 45	8.4
		7	96	963± 45	7.5
		8	96	1,491± 16	15.5
		9	96	100± 10	0.7
		10	167	1,269± 13	126.9
		11	96	541± 8	4.3
		12	96	276± 17	5.1
		13	240	2,069± 52	73.9
		14	106	333± 4	1.6
		15	239	745± 56	22.6
		16	96	2,275± 25	32.5
	II	17	168	911± 22	4.0
		18	168	2,164±195	23.8
		19	120	2,888± 83	13.3
		20	96	2,810±113	9.7
		21	96	4,756±123	16.9
		22	96	4,563±294	9.6
		23	112	4,167±139	9.1
		24	120	4,572±112	11.0
		25	96–120	All dead	.....
2.....	II	1	72	62,362±600	.....
		2	72	13,672±393	2.2
		3	76	3,033± 56	2.2
		4	123	88± 10	0.3
		5	73	121± 11	13.4
		6	136	849± 25	70.8
		7	96	1,109± 24	13.0
		8	96	2,076± 27	18.7
		9	96	845± 27	4.1
		10	96	856± 8	10.1
		11	96	601± 19	7.0
		12	96	All dead	.....
3.....	I	1	103	3,780± 83	3.8
		2	96	476± 13	1.3
		3	96	1,147± 39	23.9
		4	168	2,265± 36	19.2
		5	168	1,098± 29	4.9
		6	123	691± 13	6.3
		7	96	539± 16	7.8
		8	96	1,057± 16	19.6
		9	96	1,025± 10	9.7
		10	96	1,024± 11	10.0
		11	120	823± 38	8.0
		12	231	1,272± 19	15.5
		13	101	310± 6	2.4
		14	125	433± 11	14.0
		15	96	475± 23	11.0
		16	96	1,686± 39	35.2
		17	111	561± 19	3.3
		18	100	514± 40	9.2
		19	96	448± 13	8.8
		20	96	260± 23	5.7
		21	96	1,393± 13	53.6
		22	96	1,209± 27	8.7
		23	96	1,102± 13	9.1
		24	96	1,210± 18	11.0
		25	96	1,235± 15	10.2

* Population growth is expressed as the ratio of final to initial population.

Standard error (S.E.) = $\sqrt{\frac{(d)^2}{n(n-1)}}$.

batches of medium appear to have been more favorable than others, although, so far as could be determined, all were of identical composition.

Series 2 was lost as the twelfth transfer by contamination of the culture flasks by permanganate from the steaming vessels; series 1, at the twenty-fifth transfer, by mechanical failure of the incubator which lowered the temperature below the death point. As a result, it is impossible to determine the cause of the increased populations in transfers 17–24 of this series. However, none of them showed nitrite or nitrate contents significantly above controls. Except for these few transfers, no populations have been obtained which were comparable to those of Burrows. Series 3 has been carried beyond 30 transfers with no increase in population.

Table 2 presents the results of ten series, comparing nonabsorbent and absorbent cotton plugs and glass caps. Statistical analysis shows that, in every case, undoubtedly significantly higher populations resulted in cotton-plugged flasks. These results were not due to differences in the diffusion of gases (as measured by CO_2) past the various closures or to the deleterious effects of soft-glass caps, as shown by similar results with Pyrex caps. Moreover, replacing the cotton plug by glass after autoclaving did not reduce the population obtained. Cultures 8 and 9 showed many dividing forms at counting, indicating that the maximum population had not been reached. In none of these series was an attempt made to determine maximum population, so that it is not possible to decide whether nonabsorbent cotton is more favorable than absorbent. All cultures were analyzed for nitrite and nitrate; in every case the nitrite content of glass-covered and cotton-plugged flasks was the same, and no nitrate was detectable. These results suggest very strongly that the cotton plugs contribute some metabolizable organic material to the medium.

TABLE 2

EFFECT OF COTTON PLUGS

Culture No.	Hours Incubation	Medium	Initial Population	Final Population		
				Glass Caps	Nonabsorbent Cotton	Absorbent Cotton
1	135	I	149	1,285±45	11,267±113	.....
2	96	I	111	2,076±27	20,858±165	.....
3	96	I	96	1,491±16	21,835±287	.....
4	96	I	55	561±19	3,351±239	2,983±267
5	96	I	50	514±40	3,199±167	2,726±116
6	96	II	85	856± 8	9,229±162	.....
7	96	II	60	996±13	5,780±179	3,950±200
8	96	II	40	470± 9	1,985± 7	1,776± 96
9	96	II	30	448±13	1,170± 55	1,875±161
10	96	II	58	906± 9	4,694±236*	4,630±203*

* Sterile glass caps substituted for cotton plugs after autoclaving.

THE QUESTION OF AN INORGANIC ENERGY SOURCE

Nitrite and nitrate determinations were made on the cultures in Table 1 at the time of counting. Previous to the use of the diphenylbenzidine method, no

nitrate was detectable; during this period Shinn's original method for nitrite was used, and the amounts present were so small as to be barely detectable photometrically. With the use of more sensitive analytic methods, reliable values for nitrite and nitrate were obtained; for example, transfers 20–25 of series 3 gave nitrite levels of 0.021, 0.019, 0.020, 0.020, 0.015, and 0.025 μgm. nitrite nitrogen per milliliter; transfers 21–24 of series 1, 0.025, 0.020, 0.025, and 0.019 μgm. nitrite nitrogen per milliliter. Nitrate levels were not greater than those of uninoculated controls.

In an attempt to determine whether the chilomonads were oxidizing ammonia to produce these small amounts of nitrite, organisms grown in acetate medium with vitamins were concentrated and washed by centrifugation at one hundred to two hundred times gravity and inoculated into Media I and II to give concentrations of 10,000, 20,000, 50,000, and 100,000 per milliliter. Analyses at 96 and 192 hours showed no nitrate and only 0.01–0.02 μgm. more nitrite nitrogen per milliliter than did heat-killed controls. During incubation, all the populations declined slowly, with about 50 per cent of the organisms dead at 192 hours. Starch, but not fat, content slowly decreased also, with some organisms free of starch at 96 hours and most without starch at 192 hours. However, dead and disintegrating organisms in these cultures released organic materials into the medium which might have been used by the survivors, so that the results were not conclusive.

ATTEMPTS AT INCREASING MAXIMUM POPULATIONS

Since thiamin and possibly other B vitamins increase populations in acetate media, thiamin or Pace's vitamin mixture was added to Media I and II to a total of 5 transfers, but in no culture was there a significant increase in population. In some of these vitamin experiments, the $FeCl_3$ in both media was replaced by sterile, freshly prepared $FeSO_4$ at equivalent concentration. Again no significant increase in population occurred.

Meyerhof (1917) found an optimum ammonia concentration of 5×10^{-3} M for the nitrite bacteria. Although the ammonia concentration of Medium II was regarded as optimum by Mast and Pace, their results were obtained with acetate media and might not apply to inorganic media. Accordingly, two series of 10 cultures each, in which the initial ammonia concentration ranged from 0.89 to 8.9×10^{-3} M, were run. The maximum population was not significantly increased in any of the cultures.

The slight additional possibility remained that the chilomonads catalyze only the reaction $NO_2^- \rightarrow NO_3^-$. Preliminary experiments showed that nitrite-nitrogen levels above 20 μgm. per milliliter were unfavorable. Medium II was supplemented with $NaNO_2$ in concentrations of 1.95–18.70 μgm. nitrite nitrogen per milliliter and inoculated from transfer 28 of series 3. The cultures were analyzed for nitrite and nitrate immediately after inoculation and again 24 hours after the maximum population was reached in control cultures. There was no consumption of nitrite, formation of nitrate, or increase in maximum population.

GROWTH-CURVE STUDIES

Growth curves were run in both inorganic media and three modifications of each: (1) with 0.001 mg. per cent thiamin HCl; (2) with 250 mg. per cent $CH_3COONa \cdot 3H_2O$; and (3) with 250 mg. per cent $CH_3COONa \cdot 3H_2O$ plus 0.001 mg. per cent thiamin HCl. The resulting curves were found to coincide for both media. Figure 1 shows that growth

ceased much sooner in the inorganic medium than in the acetate solution and continued longer in the presence of both thiamin and acetate than in the absence of either. During the logarithmic phase of the curve, the slope was identical for all four cultures, indicating that the organisms were growing at the maximum rate. In the inorganic media, cessation of growth during the third day of incubation and a steady decrease in the size of the organisms and the amount of stored food after the third day suggest an early exhaustion of the available food; since there is no appreciable change in the concentration of known inorganic substances in this time, the interpretation that traces of organic compounds already present in the medium are being used for growth is supported. The curves in Figure 1 are of the shape which Schoenborn (1946) postulated for growth supported by traces of necessary organic substances in the medium.

The alternative explanation—that cessation of growth is due to exhaustion of traces of essential inorganic materials—is improbable, since much larger populations flourish in this medium when acetate is supplied; no unusual mineral requirements have been reported for the chemoautotrophic bacteria; and no increase in population in inorganic media occurred when small amounts of Zn^{++}, Cu^{++}, Co^{++}, Sn^{++}, Li^{+}, Mn^{++}, Al^{+++}, Ni^{++}, $BO_3^{\equiv}$, I^{-}, and Br^{-} were added (see Hoagland and Snyder, 1933).

It is also apparent that in the inorganic medium thiamin has no effect on either the maximum population or the length of the maximum stationary phase, in contrast to its marked effect in acetate media. The absence of other B vitamins from the medium is not a limiting factor, since Holz (1949) has shown that *Chilomonas* is capable of synthesizing them in adequate amounts.

Nitrite determinations on culture supernate at the same time as samples were withdrawn for counting showed a low and irregular increase in all four cultures during the logarithmic and maximum stationary phases, followed by an abrupt increase during the period of population decline. At 96 hours, 0.020, 0.017, 0.031, and 0.035 μgm. of nitrite nitrogen per milliliter were found in the inorganic, inorganic plus thiamin, acetate, and acetate plus thiamin cultures, respectively. At 5 days these values had increased to 0.040, 0.033, and 0.034 μgm. for the first three cultures; at 9 days the fourth culture contained 0.20 μgm. The source of this nitrite is undetermined, but, since it is produced even in the presence of acetate, it cannot be interpreted as evidence of chemoautotrophy. Nitrate tests on the culture su-

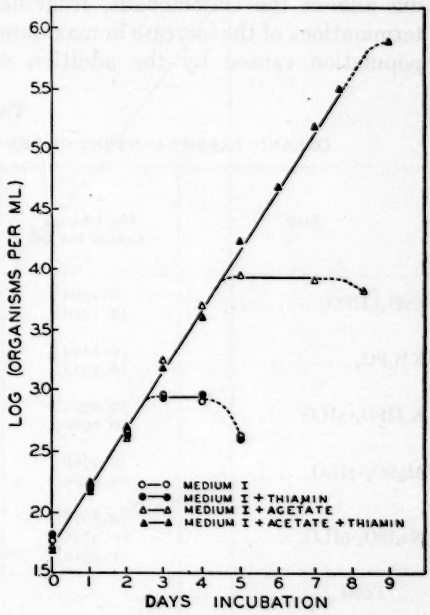


FIG. 1.—Growth curves in Medium I and three modifications. Inoculum: series 3, transfer 35. Incubated under increased CO_2 tension at 23° C.

pernate at intervals during the growth of the populations gave only the faintest perceptible blue color with diphenylbenzidine.

CARBON STUDIES

Additional evidence on the question of chemoautotrophy can be obtained from a knowledge of the organic carbon content of the uninoculated culture media and of the chilomonads, from determinations of the increase in maximum population caused by the addition of small amounts of a carbon source, and from the effect of CO_2 on growth and population levels.

Available facilities did not permit analysis of the autoclaved medium itself. Instead, analyses were made of the constituents of the media; the results are presented in Table 3. The wet combustion method used does not permit analysis of halide salts, so that the figures given are minimum values for the two media. Any organic carbon contributed by the distilled water and halide salts is not included.

Chilomonads were grown in acetate and ethanol media supplemented with vitamins, concentrated by centrifugation at approximately two hundred times gravity, suitably diluted with inorganic medium and 1.00-ml. volumes of the suspension distributed to the combustion tubes and dried *in vacuo*. Counts made on aliquots of the suspensions gave the number of chilomonads per tube, and blanks of 1.00 ml. of the diluting medium were always run. Replicate determinations gave a carbon content of 194 μgm. per 10^6 chilomonads. There was no significant difference between organisms grown in acetate and those grown in ethanol media.

Aliquots of a stock solution of $CH_3COONa \cdot 3H_2O$ were added to Medium II at pH 8.6 to give the final carbon concentrations shown in Figure 2. The cultures were incubated under increased CO_2 and the maximum populations determined. In the absence of thiamin, 1 mg. of organic carbon per liter increased the maximum population

TABLE 3*

ORGANIC CARBON CONTENT OF THE CONSTITUENTS OF THE INORGANIC MEDIA

Salt	Mg. Organic Carbon per Gm.	Average	Mg. Organic Carbon per Liter	
			Medium I	Medium II
$(NH_4)_2HPO_4$	{0.1466, 0.1390}	0.1428	0.1428	.....
KH_2PO_4	{0.1164, 0.1213}	0.1189	0.0234	.....
$K_2HPO_4 \cdot 3H_2O$	{0.0761, 0.0900}	0.0830	.....	0.0227
$MgSO_4 \cdot 7H_2O$	{0.0768, 0.0780}	0.0774	0.0155	0.0076
$Na_2SiO_3 \cdot 9H_2O$	{0.8678, 0.9741, 1.0516}	0.9645	0.2193	0.2193
Total	.....	.....	0.4010	0.2496

* All analyses corrected for carbonate-bicarbonate carbon.

to about three times that without added carbon; with thiamin present, the same amount of added carbon increased the population about eleven fold. Since this amount of carbon is only two and a half to four times the minimum content of the inorganic medium shown in Table 3, it is obvious that the growth observed in the serial transfers does not constitute evidence of chemoautotrophy. With the exception of the lower portion of curve *B*, the curves in Figure 2 show the straight-line relationship expected when exhaustion of food is the limiting factor in the growth of a population.

No attempts were made to grow *Chilomonas* in the absence of CO_2, since Jahn (1936) showed that it is at least stimulatory in organic media. A similar stimulatory effect appears in all the curves in Figure 1; these cultures were inoculated from a stock in the maximum stationary phase, which ordinarily produces an appreciable initial lag phase in subcultures. The presence of increased CO_2 in the growth-curve experiments abolished this initial lag phase.

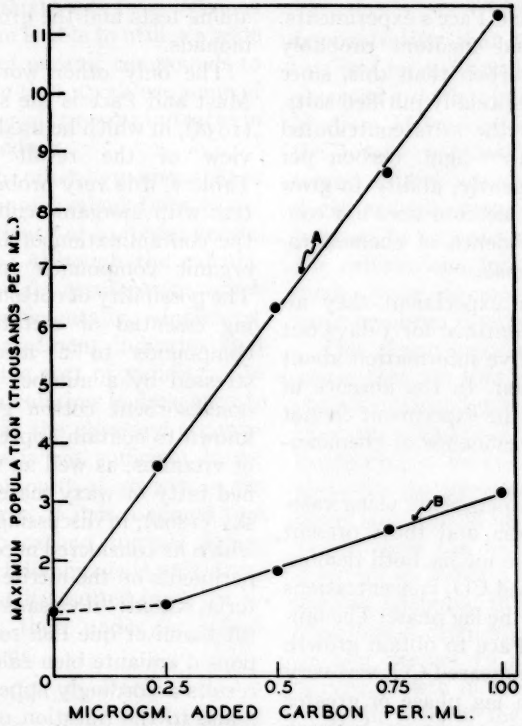


FIG. 2.—Effect of small amount of added carbon on the maximum population in Medium II. Inoculum: transfer 5 of the fourth series; initial population: 112 ± 23. Curve *A*, with exogenous thiamin; curve *B*, without exogenous thiamin.

DISCUSSION

In their original investigations, Mast and Pace (1933) used an isolation-culture technique and allowed only 24 hours of growth between transfers. Their Table 7 gives 4.00 divisions per day as the high-

est in inorganic solutions, equivalent to 80 chilomonads or 15.5×10^{-3} μgm. chilomonad carbon per milliliter. Hutchens, Podolsky, and Morales (1948) found that *Chilomonas* assimilated 50–55 per cent of added acetate, so that 15.5×10^{-2} μgm. organic carbon per milliliter should be a considerable excess over the catabolic and anabolic needs of the chilomonads in Mast and Pace's experiments. Their uninoculated medium probably contained more carbon than this, since they did not use especially purified salts, and in this study the salts contributed at least 2.49×10^{-2} μgm. carbon per milliliter. Consequently, ability to grow in their inorganic medium does not constitute reliable evidence of chemoautotrophy in *Chilomonas*.

In at least one experiment they allowed growth to continue for 3 days but gave no quantitative information about the final population. In the absence of such information, the experiment cannot be cited as good evidence of chemoautotrophy.

Jahn's (1936) experiments using caseinhydrolyzate media and these present studies with simple media both demonstrate that increased CO_2 concentrations shorten or abolish the lag phase. The failure of Mast and Pace to obtain growth in the absence of increased CO_2 was very probably due to a lag phase of greater duration than the 24 hours between transfers.

In their demonstration of nitrate production, Mast and Pace used the diphenylamine test. This reaction is not specific for nitrate and gives positive tests with a variety of oxidizing agents. Moreover, they apparently failed to test acetate solutions, in which the organisms had grown, for nitrate. No demonstration of an inorganic energy source can be acceptable unless it is shown that this source is not used in the presence of organic substrates, such as acetate, which are known to be readily utilized.

Finally, their sterility tests were performed with nutrient agar, which will not demonstrate the presence of nitrite or nitrate bacteria as contaminants. The presence of these organisms in their cultures is possible and could have accounted for both the positive diphenylamine tests and the growth of the chilomonads.

The only other work in support of Mast and Pace is the study of Burrows (1938), in which he used cotton plugs. In view of the result summarized in Table 2, it is very probable that his success with inorganic cultures was due to the contamination of the medium with organic compounds from the cotton. The possibility of cotton plugs contributing essential or metabolizable organic compounds to a medium has been stressed by a number of workers. The nonabsorbent cotton generally used is known to contain appreciable quantities of vitamins, as well as various unidentified fatty or waxy materials. Winogradsky (1890), in discussing the precautions which he considered necessary during experiments on the nitrite and nitrate bacteria, stated: "il va sans dire que le coton fut banni et que l'on se servait de tampons d'amiante bien calcinés." Burrows' results accordingly appear not to be germane to the question of chemoautotrophy in *C. paramecium*.

If traces of carbon compounds are to serve as energy and carbon sources, then there must be some assurance that *Chilomonas* will utilize at least some of them. It now seems well established that *C. paramecium* can use acetate, lactate (Hutchens *et al.*, 1948), ethanol (Pace, 1947), and pyruvate[4] as sole carbon

[4] Unpublished data of the author. Preliminary experiments indicate that no growth occurs in the absence of exogenous thiamin with pyruvate as sole carbon source; further studies of this relationship are in progress.

sources and that butyric, isobutyric, valeric, propionic, caproic, isocaproic, octylic, nonylic, myristic, and heptylic acids, and propyl, amyl, butyl, and esylic alcohols are at least stimulatory in silk-peptone media (Provasoli, 1938). Since *Chilomonas* will grow readily in various peptone media, it can also utilize some of the organic compounds present in these complex mixtures. It is obvious that the organism is able to utilize a wide enough variety of organic compounds to make it probable that it can use some of the organic materials present in traces in the inorganic medium.

Workers with other organisms have used thermodynamic calculations to determine the efficiency of bacteria, yeasts, and *Chilomonas*. Although the calculations involve certain assumptions which cannot be experimentally vindicated, their general agreement indicates that they possess some sort of validity. The same type of calculation is presented to demonstrate that the observed accumulation of nitrite is not sufficient to account for the amount of growth in the inorganic cultures. If it is assumed that all the organic carbon formed during growth of the chilomonads is anhydrous glucose and that it resulted from the reduction of CO_2, then, under the experimental conditions,

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2 \qquad \Delta F_{298} = +688{,}160 \text{ cal/mol};$$

$$[NH_4]^+ + 1\tfrac{1}{2}O_2 \rightarrow HNO_2 + H_2O + H^+ \qquad \Delta F_{298} = -57{,}300 \text{ cal/mol}.$$

From the population in the growth curve in Figure 1 we obtain

Maximum population: 737×10^3/liter = 737×10^{-3} million/liter = 143 μgm. carbon,
Initial population: 66×10^3/liter = 66×10^{-3} million/liter = 13 μgm. carbon,
Net increase in chilomonad carbon = 130 μgm. carbon.

At the time of maximum population, 0.02 μgm. nitrite nitrogen per milliliter or 20.0 μgm. per liter had been formed. The nitrogen-carbon ratio is therefore 0.15. Using the thermodynamic efficiency equation of Baas-Becking and Parks (1927), we obtain

$$\frac{14}{12} \times \frac{114{,}700}{0.15} \times \frac{100}{57{,}300} = 1{,}557 \text{ per cent efficiency},$$

demonstrating that the nitrite production was far too small to account for the observed growth. Similar calculations for Medium II have shown that only with the lowest populations could the observed nitrite accumulation have produced enough energy to account for the increase in organic chilomonad carbon. No calculations for nitrate have been made, since no more than the barest trace has been detected.

The above calculations also demonstrate that the total chilomonad carbon per liter at maximum population was less than the organic carbon content of the uninoculated medium. Similar calculations for the serial transfer populations show that, except for a few transfers, no net increase can be assumed. Since the analyses in Table 3 represent minimum carbon contents, it is probable that at inoculation there was more than enough carbon present to account for the observed growth in all the cultures.

While none of the evidence presented in this study is conclusive, the weight of it indicates that *Chilomonas* is not capable of chemoautotrophy and that earlier reports were based on faulty technical procedures.

SUMMARY

1. *Chilomonas paramecium* has been established four times on two apparently inorganic media and appears capable of indefinite serial transfer in such solutions.

2. Populations in such media are low

and variable, and the use of cotton plugs increases the populations appreciably.

3. Only very small amounts of nitrite, and no nitrate, appear in the medium during growth. Massive inocula fail to increase the amounts of either present after 96 and 192 hours incubation.

4. Supplementing the medium with nitrite, vitamins, or Fe^{++} and varying the concentration of ammonia do not increase the maximum population.

5. Growth curves show an early cessation of growth in inorganic solutions, as compared with acetate solutions.

6. Organic carbon analyses of inorganic salts show that traces of organic compounds are present in these salts, and therefore in the apparently inorganic media.

7. With the addition of small amounts of acetate to the inorganic media, there is a straight-line relationship between the maximum population and the amount of acetate added.

8. Increased CO_2 appears to be stimulatory but not essential.

9. Earlier work on this problem is reviewed in the light of the additional information now available, and it is considered improbable that *Chilomonas* is capable of chemoautotrophic nutrition.

LITERATURE CITED

BAAS-BECKING, L. G. M., and PARKS, G. S. 1927. Energy relations in the metabolism of autotrophic bacteria. Physiol. Rev., 7:85–106.

BURROWS, W. 1938. Studies on the growth of *Chilomonas paramecium* in inorganic and acetate solutions. Protoplasma, 31:20–26.

HALL, R. P.; JOHNSON, D. F.; and LOEFER, J. B. 1935. A method for counting protozoa in the measurement of growth under experimental conditions. Trans. Amer. Micr. Soc., 54:298–99.

HALL, R. P., and LOEFER, J. B. 1936. On the supposed utilization of inorganic nitrogen by the colorless cryptomonad flagellate, *Chilomonas paramecium*. Protoplasma, 26:321–30.

HOAGLAND, D. R., and SNYDER, W. C. 1933. Nutrition of strawberry plant under controlled conditions. Proc. Amer. Soc. Hort. Sci., 30:288–94.

HOLZ, G. G., JR. 1949. Unpublished thesis. New York University.

HUTCHENS, J. O. 1940. The need of *Chilomonas paramecium* for iron. Jour. Cell. and Comp. Physiol., 16:265–67.

HUTCHENS, J. O.; PODOLSKY, B.; and MORALES, M. F. 1948. Studies on the kinetics and energetics of carbon and nitrogen metabolism of *Chilomonas paramecium*. Jour. Cell. and Comp. Physiol., 32:117–41.

JAHN, T. L. 1936. Effect of aeration and lack of CO_2 on growth of bacteria-free cultures of Protozoa. Proc. Soc. Exper. Biol. and Med., 33:494–98.

MAST, S. O., and PACE, D. M. 1933. Synthesis from inorganic compounds of starch, fat and protoplasm in the colorless animal, *Chilomonas paramecium*. Protoplasma, 20:326–58.

———. 1936. Why have some investigators been unable to grow *Chilomonas paramecium* in inorganic or simple organic solutions? Science, 83: 18–19.

MEYERHOF, O. 1917. Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. III. Die Atmung des Nitritbildners und ihre Beeinflussung durch chemische Substanzen. Arch. f. d. ges. Physiol., 166:240–80.

PACE, D. M. 1947. The effects of vitamins and growth-promoting substance on growth in *Chilomonas paramecium*. Exper. Med. Surg., 5:140.

———. 1948. The utilization of ethyl alcohol as a source of carbon in *Chilomonas paramecium*. Anat. Rec., 101:730.

PARKS, G. S., and HUFFMAN, H. M. 1932. The free energies of some organic compounds. "ACS Monographs," no. 60. New York: Chemical Catalog Co. Pp. 251.

PROVASOLI, L. 1938. Studi sulla nutrizione dei protozoi. Boll. d. zool. agron. e bachicolt., 9:1–124.

SCHOENBORN, H. W. 1946. Studies on the nutrition of colorless flagellates. II. Growth of *Astasia* in an inorganic medium. Physiol. Zoöl. 19:430–42.

———. 1949. Growth of protozoa in glassware cleaned with sulphuric acid–potassium bichromate solution. *Ibid.*, 22:30–35.

SHINN, M. B. 1941. Colorimetric method for determination of nitrite. Ind. Eng. Chem., anal. ed., 13:33–35.

SNELL, F. D., and SNELL, C. T. 1936. Colorimetric methods of analysis, including some turbidimetric and nephelometric methods, Vol. 1. New York: D. Van Nostrand. Pp. 766.

VAN NIEL, C. B. 1943. Biochemical problems of the chemoautotrophic bacteria. Physiol. Rev. 23: 338–54.

VAN SLYKE, D. D., and FOLCH, J. 1940. Manometric carbon determination. J. Biol. Chem., 136: 509–41.

WINOGRADSKY, S. 1890. Recherches sur les organismes de la nitrification. Ann. Inst. Pasteur., 4:213.

THE GROWTH-FACTOR REQUIREMENTS OF TETRAHYMENA GELEII E[1]

ALFRED M. ELLIOTT
Zoölogy Department, University of Michigan

THE growth-factor needs of various strains of *Tetrahymena geleii* have stimulated considerable research in recent years, much of which has led to somewhat confusing results. For example, thiamine was shown to be essential for strain E (*Colpidium striatum*, Elliott, 1939), for strain GP (*Glaucoma piriformis*, Lwoff and Lwoff, 1937, 1938; Hall and Cosgrove, 1944), and for strain H (*Colpidium campylum*, Hall, 1942). On the other hand, Kidder and Dewey (1942, 1944) have demonstrated that strain W does not require thiamine. These authors in an earlier report indicated that pantothenic acid, nicotinic acid, riboflavin, and pyridoxine were not essential for strain W (Kidder and Dewey, 1942, 1944, 1945) but find them essential in a more recent paper (Kidder and Dewey, 1949*a*, 1949*b*). Much of this confusion has come about because certain crude extracts had to be added to the otherwise well-defined basal medium in order to obtain growth of the cells. With the recent use of the highly refined growth factor protogen, instead of crude extracts, the answers to many of these problems are coming to light. The purpose of this investigation, therefore, is to determine the precise growth-factor needs of strain E, quantitatively and qualitatively.

[1] This investigation was supported in part by a grant from the Horace H. Rackham School of Graduate Studies. The author is grateful to Merck and Company for supplying the amino acids and vitamins used in this investigation and to Dr. E. L. R. Stokstad, of the Lederle Laboratories, for furnishing the protogen, without which these experiments could not have been done.

MATERIALS AND METHODS

The experimental animal used in this investigation was *T. geleii*, strain E, formerly known as *C. striatum*. The method of handling the organisms and estimating growth was the same as that reported previously (Elliott, 1949*a*). The basal medium included amino acids, carbon sources, nucleic acid, and inorganic salts in the concentrations shown in Table 1. All media were prepared in double-distilled water, and the initial pH was

TABLE 1

BASAL MEDIUM

	μ per Milliliter
l-Arginine	600
l-Histidine	150
dl-Isoleucine	75
l-Leucine	75
l-Lysine	150
dl-Methionine	150
dl-Phenylalanine	150
dl-Serine	300
dl-Threonine	300
l-Tryptophan	150
dl-Valine	150
Dextrose	1,000
Sodium acetate	1,000
$MgSO_4$	100
K_2HPO_4	100
$CaCl_2$	50
$FeCl_3$	1.0
$Fe(NH_4)_2(SO_4)_2$	5.0
$CuCl_2$	5.0
$ZnCl_2$	0.05
$MnCl_2$	0.05
Yeast nucleic acid	100

routinely set at 7.4, which is optimum for this organism. The amino acids, growth factors, and salts were made up in stock solutions and diluted to the desired concentrations when preparing experimental media. In all cases, freshly prepared solutions of growth factors were employed, and every effort was made to prevent chemical contamination. All media underwent sterilization in the autoclave at 15 pounds for 10 minutes after it was prepared; none of the growth factors was added aseptically.

The growth factor under observation was omitted from the complete medium

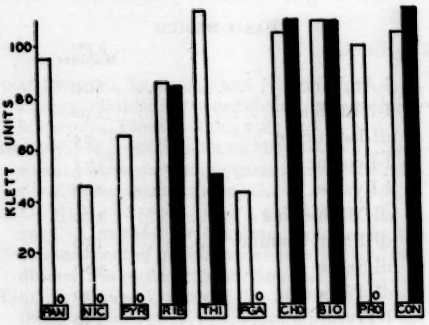


FIG. 1.—Growth is recorded in Klett units for the first (*blank blocks*) and third (*black blocks*) transfers, when the individual growth factors are omitted singly.

(basal medium plus growth factors—calcium pantothenate, nicotinamide, pyridoxine, riboflavin, thiamine, pteroylglutamic acid, choline, biotin, and protogen), and any subsequent growth was recorded through five or more transfers. In the case of essential growth factors, growth was negligible after the second or third transfer; doubtful cases, such as riboflavin and thiamine, required more transfers in order to be certain of their role in the nutrition of this cell. In the concentration series the growth factor being studied was added back to the otherwise complete medium in graded amounts, which varied with individual experiments. Each concentration level was made up in triplicate tubes; two tubes were inoculated simultaneously at the beginning of the experiment, the third was inoculated from one of the first two during the logarithmic growth phase. This procedure made it possible to check questionable cases that might arise in the first two tubes. Inoculations were made with a loop when many serial transfers were necessary to determine the essentiality of a growth factor; but in the concentration series a light inoculum of washed organisms with the pipette gave the most satisfactory results. All cultures were incubated for 96 hours in the dark at 25° C. All experiments were repeated several times, and the most consistent series are reported here.

EXPERIMENTAL

ESSENTIAL GROWTH FACTORS

Calcium pantothenate, nicotinamide, thiamine, riboflavin, pteroylglutamic acid (PGA), biotin, choline, and protogen were tested for their essentiality in the normal nutrition of strain E. All were suspected of playing some nutritional role, since they had supported full growth not only for this strain of *Tetrahymena* but for others as well. Each factor was omitted singly from the complete medium, and growth was carried through succeeding transfers until the organism failed to grow altogether or continued through a minimum of eight transfers, in which case the growth factor was considered nonessential. The results are recorded in Figure 1.

In the third transfer the ciliates failed to grow in the tubes without pantothenate, nicotinamide, pyridoxine, PGA, and protogen, some dying out in the second. Growth was maximal in the first and third and all succeeding transfers in tubes without biotin or choline; hence

these two growth factors are probably nonessential for this ciliate. While growth was not maximal in the tubes without riboflavin, it did continue at approximately half-maximal through ten transfers (at the time of the writing of this paper). It would appear, then, that riboflavin is not essential for continued growth of this organism, although it should be routinely added to the medium if full growth is desired. Growth without thiamine is maximal on the first transfer but falls off rapidly in succeeding transfers and fails altogether in the fourth or fifth. Therefore, it seems to be essential for continued culturing of the cell, an observation which supports data found earlier with a relatively crude medium (Elliott, 1939).

CONCENTRATION SERIES

In this group of experiments dose response curves were determined for each of the eight B vitamins and protogen in an effort to find the proper concentrations for optimal growth.

Calcium pantothenate.—Growth was negligible in the blank but increased progressively from concentrations of 0.0001–0.1 γ/ml, the latter supplying sufficient calcium pantothenate to produce maximal growth (Fig. 2). Half-maximal growth occurred at about 0.01 γ/ml, and concentrations higher than 0.1 γ/ml seem to be inhibitory. These experiments also confirm the essentiality of this vitamin and explain its stimulating effect, as observed in an earlier report (Elliott, 1935).

Nicotinamide.—The dose response curve for this vitamin approximates that for calcium pantothenate; half-maximal growth occurs at about 0.01 γ/ml and full growth at 0.1 γ/ml (Fig. 3). Likewise, increased amounts tend to decrease the growth. The essentiality of nicotinamide is also confirmed.

Pyridoxine.—It was necessary to employ considerably higher concentrations of this vitamin to obtain maximal yield of cells. Half-maximal growth occurs at about 1.1 γ/ml and full growth at 2.0 γ/ml (Fig. 4). Increasing the amount to 100 γ/ml seemed to have little influence on growth. Here again the essentiality of pyridoxine is confirmed.

Riboflavin.—Approximately two-thirds maximal growth occurs regularly without the addition of riboflavin; hence similar growth would be expected here in the blank tubes (Fig. 5). The addition of amounts as small as 0.001 γ/ml is sufficient to produce maximal yield. Higher concentrations may have an inhibitory effect, although this is doubtful.

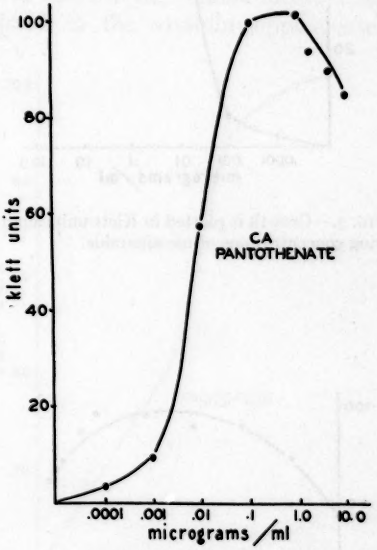


FIG. 2.—Growth is plotted in Klett units against varying concentrations of calcium pantothenate.

Thiamine.—Since maximal growth occurs without thiamine in the first transfer, it is, of course, impossible to check its growth needs for this vitamin. However, a dose response curve was done in order to note any stimulatory or inhibitory effects (Fig. 6). Concentrations higher

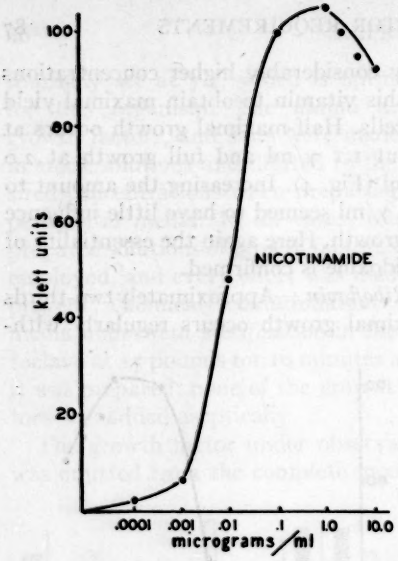


FIG. 3.—Growth is plotted in Klett units against varying concentrations of nicotinamide.

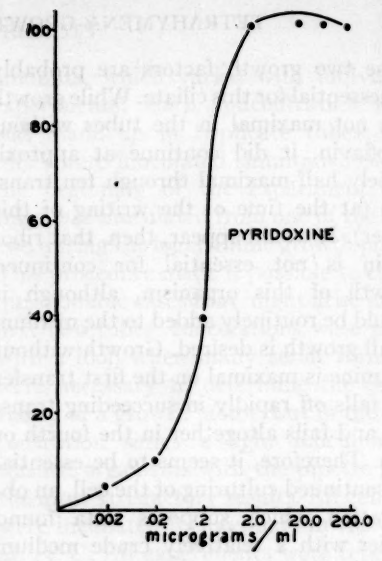


FIG. 4.—Growth is plotted in Klett units against varying concentrations of pyridoxine.

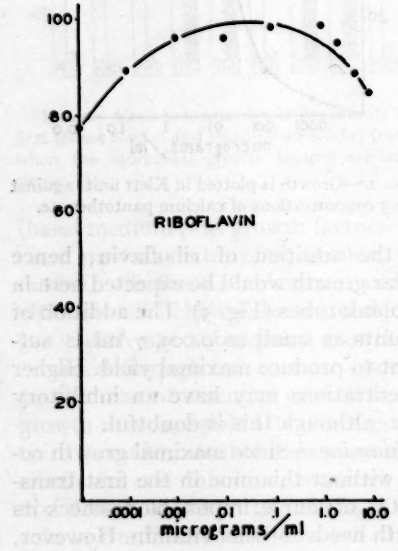


FIG. 5.—Growth is plotted in Klett units against varying concentrations of riboflavin.

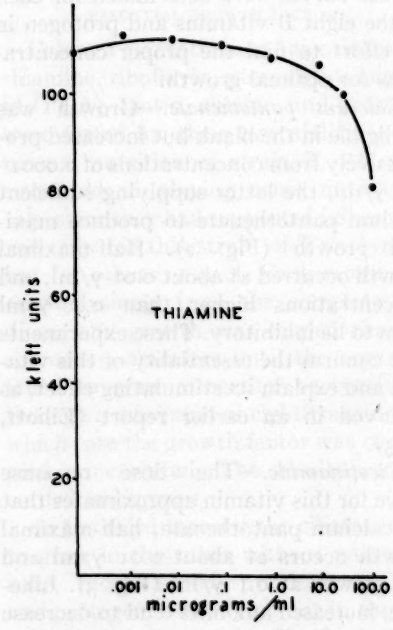


FIG. 6.—Growth is plotted in Klett units against varying concentrations of thiamine.

than 10.0 γ/ml seemed to show definite growth inhibition.

Pteroylglutamic acid.—Because of the effectiveness of this vitamin in high dilution, much lower concentrations were necessary to determine its precise quantitative needs. Half-maximal growth occurs at 0.0016 γ/ml and full growth at

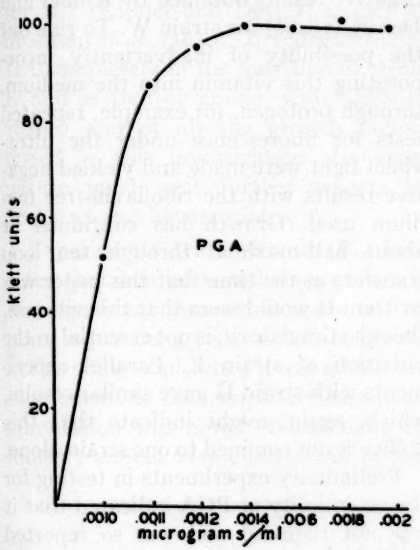


FIG. 7.—Growth is plotted in Klett units against varying concentrations of PGA.

0.0018 γ/ml (Fig. 7). Higher concentrations up to 1.0 γ/ml (not shown in Fig. 7) showed no effects on growth. A comparative series (not shown in this report) demonstrated that PGA was slightly more effective than PTGA (pteroyldiglutamylglutamic acid).

Choline and biotin.—Since neither of these growth factors influenced growth in any way, no dose response curves were necessary.

Protogen.—The dose response curve with this partially purified growth factor indicated that half-maximal growth occurs at about 0.09 unit/ml and full growth at 1.0 unit/ml, which is in substantial agreement with that recommended by Dr. E. L. R. Stokstad in a private communication.[2]

DISCUSSION

Until recently growth-factor studies on ciliates were done with highly complex media in which attempts were made to destroy the vitamin under investigation and then add it back to the medium. If growth failed in the treated medium but occurred in the vitamin-supplemented

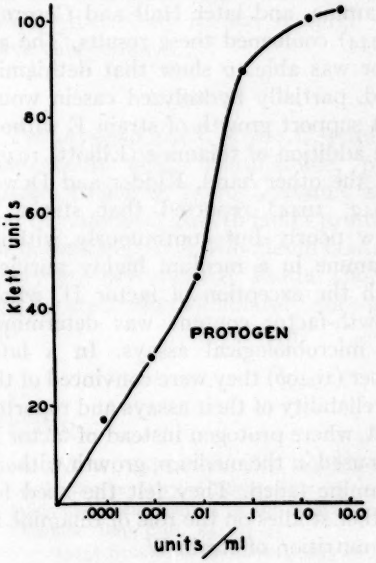


FIG. 8.—Growth is plotted in Klett units against varying concentrations of protogen.

medium, the vitamin was thought to be essential. Because of the possibility of the formation of inhibitors by such drastic treatment of the medium, some doubt has now been thrown on this work, although, perhaps, many of the conclusions were valid at the time and may still be.

[2] A standard unit of protogen, as defined by Stokstad *et al.* (1949), is the amount present in 1 mg. of liver-press-cake digest.

While most of the early work was done with various strains of *T. geleii*, Garnjobst, Tatum, and Taylor (1943) found that *Colpoda duodenaria* required pantothenic acid, riboflavin, thiamine, pyridoxine, and nicotinamide in addition to a complex bacterial plasmoptyzate. The earlier work with *T. geleii* centered around the requirement of certain B vitamins, particularly thiamine, where there was considerable confusion. For instance, Lwoff and Lwoff (1937–38) showed that strain GP (*Glaucoma piriformis*) needed thiamine, and later Hall and Cosgrove (1944) confirmed these results. The author was able to show that dethiaminized, partially hydrolyzed casein would not support growth of strain E without the addition of thiamine (Elliott, 1939). On the other hand, Kidder and Dewey (1942, 1944) reported that strain W grew poorly but continuously without thiamine in a medium highly purified, with the exception of factor II, whose growth-factor content was determined by microbiological assays. In a later paper (1949*b*) they were convinced of the unreliability of their assays and reported that, where protogen instead of factor II was used in the medium, growth without thiamine failed. They felt the need for further studies on the role of thiamine in the nutrition of strain W.

In the present investigation strain E seemed to grow for a time without thiamine but died out in subsequent transfers. Parallel experiments with strain H showed similar results, which may mean that this is not merely a strain difference. Tittler (1949) was able to show that without thiamine there was a decided drop in pH after 7 days' incubation, owing to the accumulation of pyruvic acid in the medium; these adverse conditions were responsible for the dying-out of the cells. By adding 0.01 γ/ml of thiamine, the pH remained stable, pyruvic acid did not accumulate, and the cells grew normally. Whether or not this is the role of thiamine in the nutrition of strain E, the present investigation seems to indicate that this vitamin is essential for continued growth.

The ability of strain E to grow without riboflavin was surprising, in view of the negative results obtained by Kidder and Dewey (1949*b*) for strain W. To rule out the possibility of inadvertently incorporating this vitamin into the medium, through protogen, for example, repeated tests for fluorescence under the ultraviolet light were made and yielded negative results with the riboflavin-free medium used. Growth has continued at about half-maximal through ten loop transfers at the time that this paper was written. It would seem that this vitamin, though stimulatory, is not essential in the nutrition of strain E. Parallel experiments with strain H gave similar results, which, again, might indicate that this ability is not confined to one strain alone.

Preliminary experiments in testing for the essentiality of PGA indicated that it was not required and was so reported (Elliott, 1949*a*, *b*); however, the results of this investigation definitely indicate that this vitamin is essential. These discrepancies could possibly be accounted for by assuming that the organism adapts to a PGA-free medium. Since similar phenomena have been observed with amino acids and their analogues (Elliott and Hogg, 1949), it is not at all unlikely that it could occur in this instance. This possibility is under investigation at the present time.

The nonessentiality of biotin was not expected, in view of its almost universal requirement by other organisms. The possibility of its being added as a contaminant was ruled out by adding avidin (in the form of fresh egg white) to a controlled series of experiments; growth was

as good in the tubes containing avidin as in the controls, both with and without exogenous biotin. Therefore, in so far as can be told by these experiments, it seems that biotin is not necessary in the nutrition of this ciliate.

Protogen is a highly refined extract of unknown composition isolated from factor IIA (Kidder and Dewey, 1949*c*) by Stokstad *et al.* (1949). It replaces factor IIA completely in the nutrition of strain E and is free of amino acids (Elliott, 1949*a*) and of all the growth factors tested in this investigation. Its exact nature must wait further reports by Stokstad and his associates.

Experiments with vitamin B_{12} indicated that it failed to influence growth in any significant manner. It could not replace any of the growth factors employed in this study, and, when it was added as a supplement to the complete medium, no stimulation was noted. While some inhibitory effects were observed in certain instances, they were probably insignificant.

This investigation confirms the work of others that this protozoan, like mammals, is poorly equipped with enzymes that will allow it to synthesize protoplasm from simple food sources. Its long dependence on bacteria as a source of food has tended toward a gradual loss in synthetic ability. The indication of adaptive power is a sign that perhaps this loss can be reversed to some extent, so that the cell can once again survive on a simpler diet. Only further research will solve this problem.

SUMMARY

Tetrahymena geleii, strain E, requires pantothenic acid, nicotinamide, pyridoxine, thiamine, pteroylglutamic acid, and the unknown growth factor protogen for normal nutrition. Riboflavin is stimulatory but not essential. Biotin, choline, and B_{12} are neither stimulatory nor essential.

LITERATURE CITED

ELLIOTT, A. M. 1935. The influence of pantothenic acid on growth of Protozoa. Biol. Bull., **48**:82–92.

———. 1939. The vitamin B complex and the growth of *Colpidium striatum*. Physiol. Zoöl., **12**: 263–73.

———. 1949*a*. The amino acid requirements of *Tetrahymena geleii* (E). *Ibid.*, **22**:337–45.

———. 1949*b*. The growth factor requirements of *Tetrahymena geleii E*. Anat. Rec. (abstr.), **105**: 125.

ELLIOTT, A. M., and HOGG, J. F. 1949. Comparative amino acid metabolism of *Tetrahymena geleii E*. Anat. Rec. (abstr.), **105**:125.

GARNJOBST, L.; TATUM, E. L.; and TAYLOR, C. V. 1943. Further studies on the nutritional requirements of *Colpoda duodenaria*. Jour. Cell. and Comp. Physiol., **21**:199–212.

HALL, R. P. 1942. Incomplete proteins as nitrogen sources and their relation to vitamin requirements in *Colpidium campylum*. Physiol. Zoöl., **15**:95–107.

HALL, R. P., and COSGROVE, W. B. 1944. The question of the synthesis of thiamine by the ciliate, *Glaucoma piriformis*. Biol. Bull., **86**:31–40.

KIDDER, G. W., and DEWEY, V. C. 1945*a*. Studies on the biochemistry of *Tetrahymena*. IV. Amino acids and their relation to the biosynthesis of thiamine. Biol. Bull., **89**:131–43.

———. 1945*b*. Studies on the biochemistry of *Tetrahymena*. VI. Riboflavin, pantothen, biotin, niacin, and pyridoxine in the growth of *T. geleii W*. *Ibid.*, **89**:229–41.

———. 1949*a*. Studies on the biochemistry of *Tetrahymena*. XII. Pyridoxine, pyridoxal, and pyridoxamine. Arch. Biochem., **21**:58–65.

———. 1949*b*. Studies on the biochemistry of *Tetrahymena*. XIII. B vitamin requirements. *Ibid.*, **21**:66–73.

———. 1949*c*. Studies on the biochemistry of *Tetrahymena*. XI. Components of factor II of known chemical nature. *Ibid.*, **20**:433–43.

LWOFF, A., and LWOFF, M. 1938. La Specificité de la neurine, facteur de croissance pour le cilie *Glaucoma piriformis*. Compt. rend. Soc. biol., **127**:1170–72.

TITTLER, I. A. 1949. The role of thiamine in the metabolism of *Tetrahymena geleii*. Anat. Rec. (abstr.), **105**:21.

THE NUTRITION OF THE MEALWORM, TENEBRIO MOLITOR L. (TENEBRIONIDAE, COLEOPTERA)[1]

G. FRAENKEL,[2] WITH THE ASSISTANCE OF M. BLEWETT AND M. COLES

Department of Zoölogy and Applied Entomology, Imperial College of Science and Technology, London, and Department of Entomology, University of Illinois, Urbana, Illinois

THIS account of the food requirements of the yellow mealworm, *Tenebrio molitor*, forms part of a series of investigations on the dietetics of insects which feed in stored products, and belonging to the genera *Tribolium*, *Plinus*, *Lasioderma*, *Sitodrepa*, *Dermestes*, *Ephestia*, *Plodia*, and *Tineola* (Fraenkel and Blewett, 1943*a*, *b*, and *c*; 1944*a*; 1946*a* and *b*; 1947). The aim of these investigations has been to devise optimal synthetic diets in which every constituent was known and thereby to determine the basic food requirements. Work on the nutrition of the mealworm has occupied us for over 5 years, but publication was delayed through the failure, for a long period, to grow *Tenebrio* successfully on an artificial diet. It was soon found that *Tenebrio* grew well on certain incompletely defined artificial diets; in addition to casein, glucose or starch, cholesterol, and salts, these also contained yeast as a source of B vitamins. A diet in which yeast was replaced by a mixture of the then known B vitamins plus the water-insoluble fraction of yeast had proved an excellent medium for most of the insects mentioned above but was found seriously deficient for *T. molitor*, as the larvae invariably died when reaching the age of 4–6 weeks. Subsequently, the need of *Tenebrio* for folic acid (pteroylglutamic acid) was established, and only then did it become clear that *Tenebrio* required yet another factor in addition to all the known B factors. This new factor we tentatively termed "B_T" (Fraenkel and Blewett, 1947; Fraenkel, Blewett, and Coles, 1948). These later discoveries made it necessary to reinvestigate some of the earlier work. The present paper deals mainly with the general aspects of the nutrition of *Tenebrio*, excepting more recent results about folic acid and B_T, which have already been published (Fraenkel and Blewett, 1947; Fraenkel, Blewett, and Coles, 1948) or are in the course of publication.

[1] The authors wish to thank the Medical Research Council of Great Britain for a grant for the study of insect nutrition, of which this investigation is a part.

[2] Contribution No. 293 from the Department of Entomology, University of Illinois, Urbana, Illinois.

MATERIALS AND METHODS

Tenebrio is much larger than the other insects from stored products already investigated and can easily be handled and weighed singly or in small groups at frequent intervals during the growing period. In this investigation we were dealing exclusively with larval growth—to be exact, with growth from the newly hatched larva, whose weight was about 0.5 mg., to the size of 60–100 mg. Each test was performed with 20 or 40 larvae, divided into two groups of 10 or 20, respectively. Each such group was grown on 3 or 6 gm. of food, respectively, in a 2-ounce wide-mouth bottle. This quantity was sufficient for the larvae to reach a weight of about 60 mg. at the age of 10–12 weeks. The quantity of food was doubled in cases when larvae were reared to the pupal stage, the pupae weighing

120–50 mg., or when larger numbers were used in a test.

Most of the tests were performed in a room the temperature and humidity of which were kept constant at 25° C. and 70 per cent relative humidity (R.H.). But in some of the later tests the temperature was 30°, with the humidity remaining at 70 per cent. Raising the temperature to 30° has the advantage of a considerable speed-up in development, without any ill effect, while a raised humidity, up to 85 per cent, also increases the growth rate but favors contamination by mold and other microörganisms.

The synthetic diet which was commonly used was that described previously (Fraenkel and Blewett, 1947). It consisted of the ingredients listed in the accompanying tabulation, with the addition of a supply

	Parts
Casein (vitamin-free "Labco")	20
Glucose (anhydr.)	80
Cholesterol	1
Salt mixture (McCollum's No. 185)	2
Water-insoluble fraction of yeast	2.5
Water	10

of vitamins of the B complex. In earlier tests the vitamin requirements were met by the addition of 5 or 10 parts of dried brewer's yeast. Yeast was later replaced by 2½ parts of the water-insoluble fraction of yeast—i.e., yeast exhaustively extracted with boiling water—and a mixture of the vitamins of the B complex given in the accompanying tabulation,

	μgm/Gm
Thiamin	25
Riboflavin	12.5
Nicotinic acid	25
Pyridoxin	12.5
Pantothenic acid	25
Pteroylglutamic acid	2.5
Choline	500
Inositol	250
Para-aminobenzoic acid (in earlier tests only)	25

expressed in micrograms per gram of the dry diet. The water-insoluble yeast fraction was used chiefly as a source of biotin and was omitted in all tests in which the effect of biotin was being investigated.

After the need of the *Tenebrio* larva for an additional factor in yeast, B_T, was discovered, this ingredient was regularly supplied in minimal quantities of a charcoal-treated yeast or liver extract or in more purified fractions to be described elsewhere (Fraenkel, 1950). As a rule, the vitamins of the B complex were added to the diet dissolved in half the quantity of water, while the B_T concentrates and vitamins to be tested for were added in the rest of the water.

In the preparation of the diets, the glucose, casein, salt mixture, and cholesterol were first mixed in the proportions stated above and weighed into the test bottles in quantities of 3 or 6 gm. Mixtures of vitamin solutions, B_T concentrates, and other water-soluble additions were pipetted into the dry basic diet and mixed in with a spatula. The diets were transferred to the constant-temperature-humidity chamber for 2 days and then ground in a small hand mortar. Subsequently, first-stage larvae which were not yet colored were added and left to grow undisturbed for an initial period of 3–4 weeks, when they had reached a weight of 3–4 mg. on an optimal diet. Henceforth, larvae from any one bottle were counted and weighed in groups at weekly intervals until the age of 10 weeks or longer, according to the design of the experiment. When larvae reached the weight of about 100 mg., growth was progressively slowed down, and before pupation, at 120–50 mg., weight was actually lost. For these reasons and also because of a relatively high variability in the growth rate as between different individuals, weight averages of over 100

mg. were not considered as a useful indication of the efficiency of a diet.

EXPERIMENTS

THE EFFECT OF DIFFERENT HUMIDITIES ON THE GROWTH RATE

Tenebrio, like insects from stored products investigated by us previously (Fraenkel and Blewett, 1943*d*, 1944*b*), grew very much faster on flour in equilibrium with 70 per cent relative humidity than at lower humidities (Fig. 1). At 13 per cent relative humidity, growth was almost at a standstill, but it has recently been shown by Leclercq (1948*c*) that relatively large quantities of food are eaten at such low humidities. The explanation for this reduction in growth at low humidities has been found in other insects from stored products to be in the utilization of part of the food for the production of metabolic water (Fraenkel and Blewett, 1944*b*). More food is eaten at low humidities in relation to increase in body weight than at high humidities. This phenomenon has also now been demonstrated for *T. molitor* (Chayen and Fraenkel, unpublished). At humidities above 70 per cent the growth rate is increased still more markedly. Such high humidities are, however, not practical in nutritional research because the diets frequently develop mold.

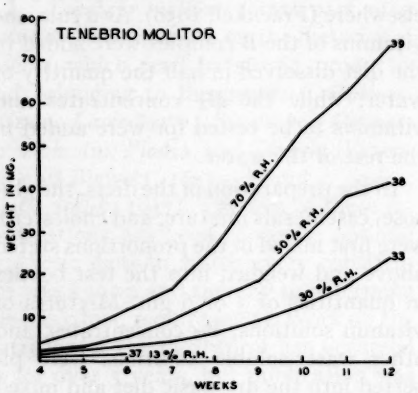


FIG. 1.—Growth of *Tenebrio* larvae, expressed by average weights, on whole-meal flour plus 5 per cent yeast at different relative humidities—13, 30, 50, and 70 per cent. Numbers of surviving larvae, out of 40, are given for the end of the experiment. Temperature 25° C.

REQUIREMENTS OF CARBOHYDRATES AND PROTEIN

Tenebrio required a high proportion of carbohydrates in the diet. In the absence of carbohydrates, growth was almost at a standstill. It was very slow with only 20 per cent of carbohydrates, faster with 50 per cent, and faster still with 75 per cent (Fig. 2). There seemed to be no significant difference between the effect of glucose and that of cornstarch. In these tests the diets contained 10 per cent of dried brewer's yeast, 1 per cent cholesterol, 2 per cent salt mixture, and varying proportions of casein and glucose or starch. Subsequently, artificial diets for *Tenebrio* always were composed on the

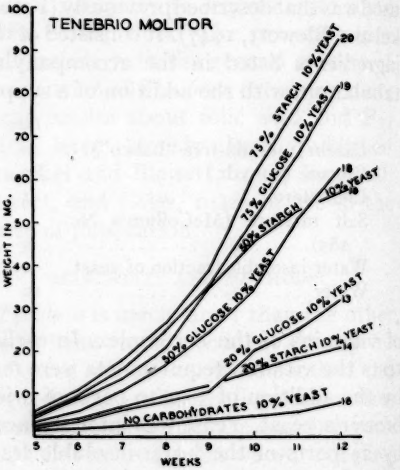


FIG. 2.—Growth of *Tenebrio* larvae on artificial diets which contain 10 per cent yeast and varying amounts of glucose or starch as well as casein. Temperature 25° C., relative humidity 70 per cent. Numbers of survivors, out of 20, are given for the end of the experiment.

basis of 80 parts of glucose to 20 parts of casein.

In a subsequent series of tests an attempt was made to determine the effects of very small amounts of protein and the lowest amount of casein which still gave an optimal effect. In these tests it was necessary to use a diet which was entirely devoid of yeast and yeast fractions in order to avoid the introduction of yeast proteins. The artificial diet described on page 93 was used, with biotin in place of insoluble yeast and with a minimal amount of a B_T concentrate, which could not have contained any proteinous material. With 10 per cent casein in the diet, growth was still nearly optimal; but, with 5, 2, and 1 per cent the growth rate was progressively slowed down, with almost no growth at 1 per cent (Table 1).

The effect of a number of different proteins, all used in quantities of 20 per cent, is also shown in Table 1. Lactalbumin (General Biochemicals, Chagrin Falls, Ohio) and peanut protein (Imperial Chemical Industries, Ltd., Explosion Division, Stevenston, Ayrshire, Scotland) had about the same effect as casein. On soy protein unheated (Archer Daniels Midland Co., Minneapolis, Minn.), growth was exceedingly slow, but there was a great improvement when the protein was preheated. This clearly indicates that the soybean inhibitor (Kunitz, 1947) also affects *Tenebrio*. With gliadin or zein, growth was virtually at a standstill. This was almost certainly due principally to the deficiency in gliadin of lysine and in zein of lysine and tryptophane, as was demonstrated for *Tribolium* (Fraenkel, 1948) and also for *Tenebrio* (Leclercq, 1948*a*). Growth on enzymatic hydrolyzates of casein and lactalbumin was very inferior, when compared with the whole protein. These diets contained starch in place of glucose, because of the frequent occurrence of chemical reactions between glucose and amino acids. The unfavorable effects of these diets could, however, not possibly have been due to the starch, because growth on a starch diet with 20 per cent casein was somewhat better than on a glucose diet. It is not possible, without further investigation, to decide whether this disadvantage of hydrolyzates is due to destruction of amino acids, though this would seem improbable in the case of enzymatic digestion, or to the change in physical consistency of the diet, brought about by the hygroscopic properties of the hydrolyzates. It should be noted that on this occasion the optimal natural diet —whole-meal flour plus 5 per cent yeast —gave a significantly better result than did the artificial diets.

TABLE 1

EXPERIMENT 506: THE EFFECT OF VARIOUS PROTEINS AND OF GRADED DOSES OF CASEIN ON GROWTH OF THE *Tenebrio* LARVA

Average weights, in milligrams, are given after 10 weeks at 30° C. and 70 per cent R.H. The diets are compounded on the formula given on page 93 but contain biotin in place of insoluble yeast fraction. In the cases of protein quantities below 20 per cent the deficit is made up with glucose.

Ingredients		Mg.
Whole-meal flour plus 5% yeast		100.3
20% casein (insoluble yeast in place of biotin)		69.1
1% casein		7.3
2% casein		14.4
5% casein		35.5
10% casein		53.7
15% casein		64.5
20% casein		63.7
20% peanut protein		60.2
20% soybean protein, unheated		13.6
20% soybean protein, heated		46.5
Lactalbumin		75.1
Gliadin		1.5
Zein		0.72
20% casein hydrolyzate (enzym. digest)	starch instead of glucose	8.3
20% lactalbumin hydrolyzate (enzym. digest)	starch instead of glucose	2.4
20% casein	starch instead of glucose	81.2

REQUIREMENTS OF STEROLS AND FAT

The requirements of *Tenebrio* of sterols have so far not been worked out in any detail, but it is obvious from the data in Figure 3 that growth is very slow in the absence of cholesterol in a diet which consisted of casein (20), glucose (80), salt mixture (2), and 5 parts of dried brewer's yeast. Half the larvae died before reaching the age of 13 weeks. Little growth took place thereafter, and all the

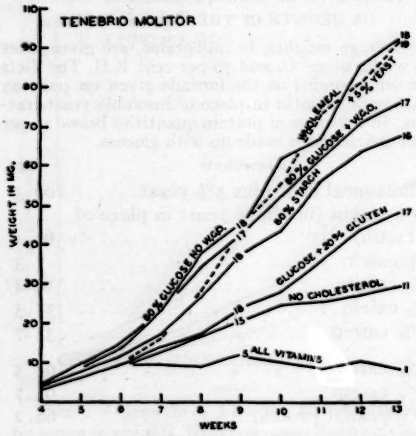


FIG. 3.—Growth of *Tenebrio* larvae in the presence and absence of wheat-germ oil (W.G.O.), with gluten in place of casein or in the absence of cholesterol. All diets contain 5 per cent yeast, except one (all vitamins), where yeast is replaced by a mixture of the members of the vitamin B complex, as listed on p. 93. Numbers of survivors, out of 20, are given after 9 and 13 weeks. Temperature 25° C., relative humidity 70 per cent.

larvae were dead by the twentieth week. The diet contained small amounts of sterols in the yeast component, and it is probable, by analogy with other insects (Fraenkel and Blewett, 1943*c*), that growth would entirely cease in the complete absence of sterols on an otherwise suitable diet.

The effect of additions of fat, supplied as wheat-germ oil in quantities of 1 per cent of the diet, was investigated on several occasions, and there has never been any indication of a beneficial action on growth or survival (Fig. 3). In this respect *Tenebrio* resembles all the other insects from stored products previously investigated by us, with the exception of *Ephestia*, where the adult moth fails to emerge from the pupal case in the absence of linoleic acid (Fraenkel and Blewett, 1946*c*).

THE FOOD VALUE OF FLOURS OF DIFFERENT EXTRACTION

Tenebrio, as a pest in flour mills, granaries, and elevators, normally lives on cereal products. It was therefore supposed that, like *Tribolium*, which also is a tenebrionid beetle, and other flour in-

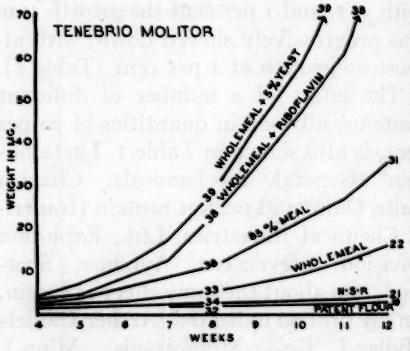


FIG. 4.—Growth of *Tenebrio* larvae on wheat flour of different rates of extraction and on wholemeal flour with the addition of yeast or riboflavin. *N.S.R.* = National Straight Run, a flour of approximately 72 per cent extraction. Numbers of survivors are given after 8 and 12 weeks. Temperature 25° C., relative humidity 70 per cent.

sects, whole-wheat flour would constitute a favorable diet. However, this was not found to be the case. Growth proceeds at a very slow rate on whole-meal flour but is greatly accelerated by the addition of 5 per cent yeast (Fig. 4). On a series of wheat flours of different extraction the rate of growth increased with the rate of extraction. On 85 per cent extraction flour, the official "National Flour" of wartime Great Britain, growth was similar to

that on whole meal, whereas a 72 per cent extraction flour ("National Straight Run," used in Great Britain during the early stages of the war) or a patent flour of probably about 60 per cent extraction hardly made possible any growth at all. In the experiment to which Figure 4 refers, 85 per cent extracted flour was actually more efficient than whole meal during the early stages of growth. These two flour fractions did not originate from the same grist, and it seems possible, from what is pointed out below, that the grain from which this 85 per cent flour was derived might have had a higher initial riboflavin content.

TABLE 2

EXPERIMENT 377: *Tenebrio molitor*

Growth on whole-wheat flour alone and with the addition of yeast, a mixture of the known B vitamins or casein, and on weatings. Numbers surviving out of 20 and average weights after 10 weeks at 30° C. and 70 per cent R.H.

	No.	Weight (Mg.)
Whole-wheat flour	11	27.1
Same+1% yeast	16	104.5
Same+B vitamins	13	70.6
Same+B vitamins+10% casein	16	66.5
Weatings	13	51.5

In another series of tests it was shown that development on weatings was much faster than on whole meal (Table 2). This result could have been anticipated from the results obtained with the white flour and whole meal. Weatings constitute a fraction which is separated from whole meal in the manufacture of white flour and therefore contain the material of nutritional importance in which white flour is specifically deficient.

The fact that relatively small quantities of yeast, even as low as 1.0 per cent (Table 2), greatly improved the efficiency of flour and that weatings proved superior as a food to whole meal suggested a deficiency of vitamins of the B complex in the flour. It is well known that, of these vitamins, riboflavin occurs in relatively small amounts in wheat and therefore the effect of adding riboflavin to flour was first investigated. The addition of riboflavin to whole meal always had the effect of speeding up the growth rate very considerably. On one occasion at least (Fig. 4) the effect of riboflavin was nearly equal to that of yeast. In the case of an 85 per cent flour, to be discussed below (Table 3), the effect of riboflavin, though significant, was less pronounced and much inferior to that of yeast. On a further occasion (Table 2) a mixture of all the known B vitamins (p. 93) was added to whole-meal flour and found to have about the same effect as was recorded on other occasions with riboflavin alone. It therefore appears that, of the known vitamins of the B complex, riboflavin is the only one in which whole-meal flour is deficient for *Tenebrio*. Quantitative differences in results must be principally due to variation in composition of the various samples of whole-meal

TABLE 3

EXPERIMENT 347: COMPARISON OF THE EFFECT OF ADDED RIBOFLAVIN IN GRADED DOSES ON A SYNTHETIC DIET

As described on page 93, but without riboflavin, and on wheat flour of 85 per cent extraction ("National Flour"). Average weights after 10 weeks and numbers surviving out of 40, at 25° C.

Addition to Diet	Synthetic Diet		85 Per Cent Flour	
	No.	Mg.	No.	Mg.
None	5	2.4	33	9.1
0.5 μgm/gm riboflavin	22	3.8	38	22
1 μgm/gm riboflavin	37	23.3	40	24.5
2 μgm/gm riboflavin	38	38.9	39	24.0
4 μgm/gm riboflavin	38	39.6	40	27.8
8 μgm/gm riboflavin	37	43.4	.....	.....
1% yeast	37	47.3	.....	.....
5% yeast	.....	.....	40	70.3

or 85 per cent flour used in these experiments.

In a further attempt to account for the superior effect of yeast over that of a mixture of pure vitamins, 10 per cent casein was added to a whole meal supplemented with vitamins, with no effect (Table 2). It therefore seems improbable that flour is deficient for *Tenebrio* in a protein component. This still leaves open the question of why 1 per cent yeast should have an effect so significantly superior to that of greatly larger quantities of pure vitamins than are contained in that amount of yeast, and it seems probable that yeast contains additional factors of importance in this particular test. There is, however, also the possibility that yeast merely increases the food consumption.

This striking effect of riboflavin suggested the possibility of using *Tenebrio* as a testing subject for assaying riboflavin in flour. A sample of 85 per cent flour was used whose content of riboflavin had been determined by chemical and microbiological methods to range between 0.5 and 0.9 μgm/gm (unpublished report of the Medical Research Council of Great Britain). In our tests the effect of graded doses of riboflavin in a synthetic diet of the composition stated on page 93 was compared with the effect of the same doses in flour (Table 3). The synthetic diet contained a minimal quantity of a liver extract as a source of B_T. This extract had been exhaustively treated with charcoal and, to the best of our knowledge, was free from riboflavin (El Sadr, Macrae, and Work, 1940).

With the synthetic diets, the addition of 2 μgm/gm of riboflavin produced an optimal effect, while with the flour an optimal effect seems to have been produced with the addition of between 0.5 and 1 μgm/gm. Unfortunately, in these tests the effect of ample amounts of riboflavin was greatly inferior to that of yeast. The data which were obtained can be best interpreted by assuming 1.5 μgm/gm of the diet as the minimal optimal riboflavin requirement of *Tenebrio* and a riboflavin content of the sample of flour of 0.75 μgm/gm, a result which agrees well with data obtained by chemical and microbiological methods.

REQUIREMENTS OF VITAMINS OF THE B COMPLEX

The requirements of *Tenebrio* larvae of vitamins of the B complex were investigated by methods already described in earlier publications (Fraenkel and Blewett, 1943*b*, 1944*a*, 1946*b*, 1947). In most experiments, except those in which the effect of biotin was investigated, the diets contained $2\frac{1}{2}$ per cent of insoluble yeast fraction. Our first attempts to grow *Tenebrio* on synthetic diets antedate 1947, before pteroylglutamic acid had been discovered and before the need of an additional factor, B_T, had been recognized. On such diets, growth was always very slow, and the larvae died at an early stage. Figure 3 gives a typical example of such a test. When pteroylglutamic acid was later included in the diets, it was found that the larvae now died even more quickly than before (Fraenkel and Blewett, 1947, Fig. 6). A factorial analysis was made of the B vitamin requirements in such a diet which was lacking in pteroylglutamic acid and B_T. With vitamins of the B complex omitted from such a diet, one at a time, it was clearly shown that virtually no growth took place in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, or pantothenic acid and that growth was depressed in the absence of choline or biotin (Fig. 5). In the presence of all these factors, growth was markedly faster, but the mortality was very high—in fact, much higher than in tests where thiamin, nicotinic acid, bio-

tin, or choline was omitted (Fig. 5; Table 6, col. A). This was presumably due to the higher requirements of B_T in cases where the larvae had reached a larger size.

This failure to obtain normal growth and survival on a diet which contained all the then known vitamins of the B complex was very puzzling at the time, and an attempt was made to get around this difficulty by growing the larvae for flavin, nicotinic acid, pyridoxin, and pantothenic acid proved limiting factors for growth. In each case growth proceeded for a period of about another 4 weeks, being maintained presumably by stores of vitamins that had accumulated during the first 4-week period. By the eighth week a pleateau was reached, and growth came more or less to a standstill. The absence of choline had only a slight depress-

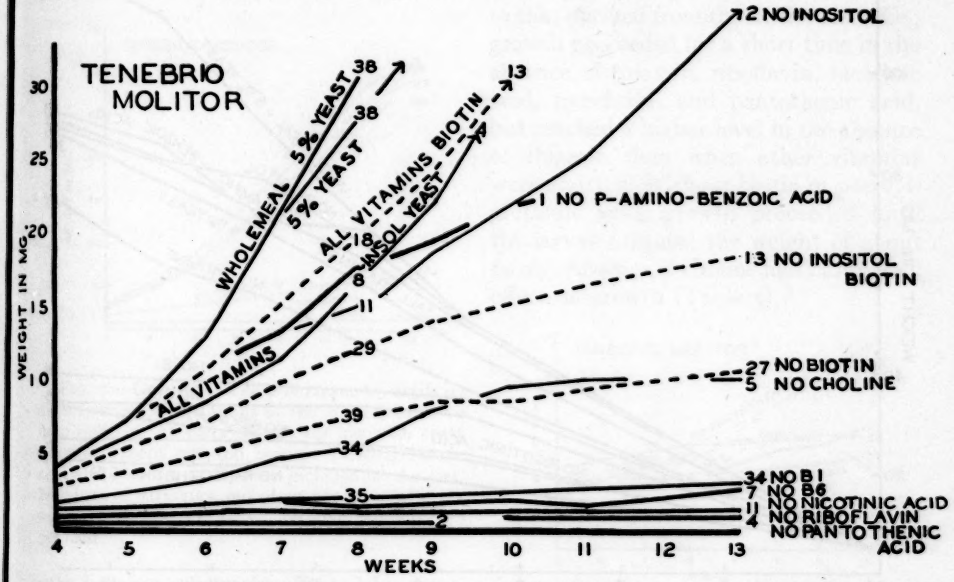


FIG. 5.—Growth of *Tenebrio* larvae on artificial diets, described on p. 93, from which vitamins of the B complex have been left out. Drawn lines refer to diets which contain insoluble yeast fraction, broken lines to diets in which insoluble yeast is absent or has been replaced by biotin. Numbers of survivors, out of 40, are given after 8 and 13 weeks. The diets contain neither pteroylglutamic acid nor a source of B_T. Temperature 25° C., relative humidity 70 per cent.

an initial period of 4 weeks on an optimal diet of whole-meal flour plus 5 per cent yeast and then transferring them to the experimental diets (Fig. 6). This new procedure proved partly successful, in that the onset of the mortality was very much delayed and did not occur until the larvae on control diets, which contained all the important vitamins, had already grown at a nearly normal rate to a size of 80–100 mg. Here, again, thiamin, riboing effect on growth, but there was no noticeable difference in growth whether inositol or para-aminobenzoic acid was present or absent, and the mixture of all the vitamins proved as successful as 5 per cent yeast.

The mortality in these tests again shows a significant trend. At the end of the experiment the larvae had started to die in the presence of all vitamins, but there was no mortality in the absence of

either thiamin, nicotinic acid, or pyridoxin where growth had been so much slower.

This experiment was repeated again after the need of *Tenebrio* for pteroylglutamic acid and the new factor, B_T, had been discovered (Fraenkel and Blewett, 1947). Pteroylglutamic acid was added to the mixture of vitamins to be tested, and a source of B_T was supplied in what was considered the minimum quantity of a liver-charcoal filtrate, corresponding to about 0.6 per cent of dried liver solids. This diet proved in the end to have been somewhat deficient in B_T, as there was a slight mortality in the complete diet, there being 27 survivors out of 40 after 12 weeks. There could, however, have been no doubt about the relative importance of the single components of the vitamin supply (Figs. 7 and 8). Virtually no growth occurred in the absence of either thiamin, riboflavin, nicotinic acid, pyridoxin, or pantothenic acid. It is doubtful whether the slightly superior growth in the absence of pantothenic acid is of any significance, especially as the mortality was very high. The absence of pteroylglutamic acid or biotin allowed slow growth to take place. There was no indication in this experiment that the absence of choline, inositol, or para-aminobenzoic acid had any effect on the growth rate.

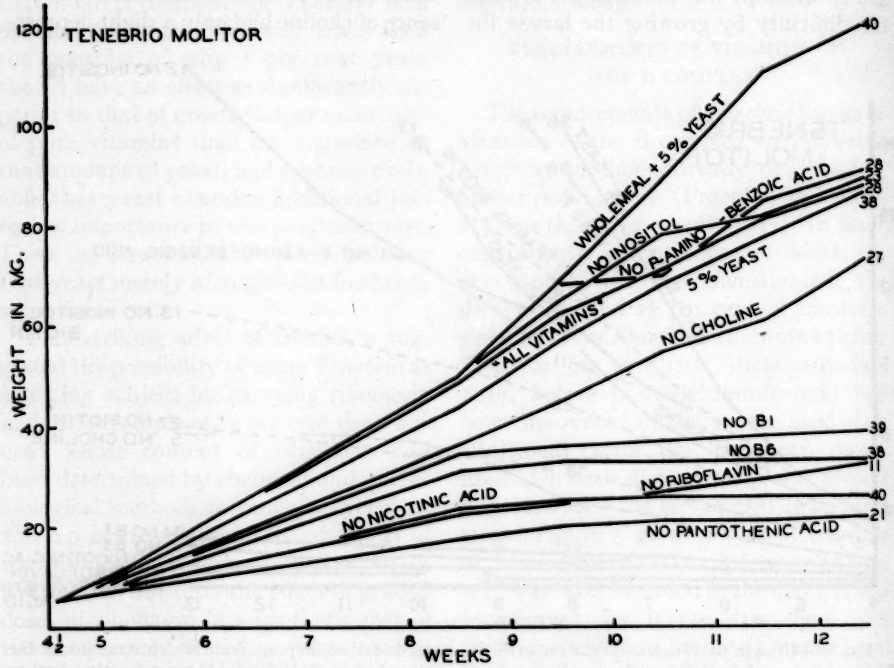


FIG. 6.—Growth of *Tenebrio* larvae on diets deficient in a single member of the vitamin B complex. The larvae were grown at first for 4½ weeks on whole meal flour plus 5 per cent yeast and were then transferred to the artificial diets. Numbers of survivors, out of 40, are given after 12½ weeks. The diets contain neither pteroylglutamic acid nor a source of B_T. Temperature 25° C., relative humidity 70 per cent.

Subsequent to the results so far described, progress was made in the purifi-

cation of B_T, and it was considered desirable to investigate the qualitative and quantitative requirements of the B vitamins in a diet which contained a minimal quantity of a purified B_T concentrate. The total amount of solids in this B_T concentrate was about 12 μgm/gm of the diet. In this experiment the number of larvae was doubled to 20 per bottle. The result is given for the seventh week of the tests, because subsequently the growth

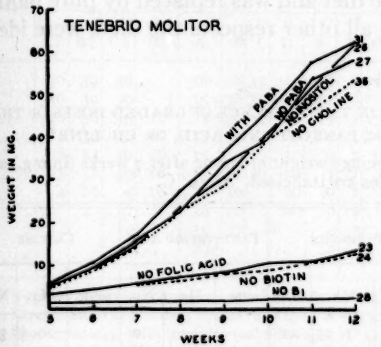


FIG. 7.—Growth of *Tenebrio* larvae on artificial diets from which vitamins of the B complex have been omitted. All diets contain the insoluble yeast fraction, except one (no biotin). Pteroylglutamic acid and a source of B_T were included in the diet. Numbers of survivors, out of 40, are given after 12 weeks. Temperature 25° C., relative humidity 70 per cent.

rate became adversely affected by overcrowding. Clear-cut results about the threshold requirements of six B vitamins were obtained (Table 4), the following amounts representing minimal quantities which gave an optimal effect:

	μgm/Gm
Thiamin	1
Riboflavin	8
Nicotinic acid	16
Pyridoxin	2
Pantothenic acid	8
Choline	300

At the same time, we investigated the possibility of obtaining larvae which were deficient in a particular vitamin of the B complex but were of larger size than those which had been grown on a deficient diet from the beginning and were too small for dissection studies. An experiment was therefore performed on lines similar to that described above (Fig. 6), where larvae were grown for an initial period of 4 weeks on whole-meal flour plus 5 per cent yeast and then transferred to diets which were deficient in a single vitamin. The result conformed to that derived from the earlier tests, i.e., growth proceeded for a short time in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, and pantothenic acid, but reached a higher level in the absence of thiamin than when other vitamins were omitted. Without biotin or pteroylglutamic acid, growth proceeded until the larvae attained the weight of about 60 mg. Absence of choline had hardly any effect on growth (Table 5).

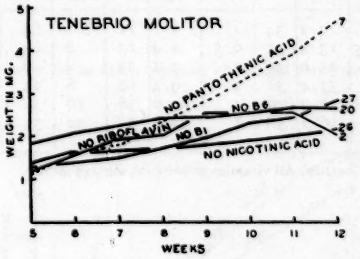


FIG. 8.—Refers to the same test as Fig. 7. Growth in the absence of five vitamins is shown on a larger scale in the ordinate.

Little has been said so far about the mortality of larvae kept on diets deficient in one particular vitamin. Table 6 summarizes the larval mortality in four different experiments that have already been referred to above. These figures cannot be compared strictly, for the reason that the conditions in the four different series of tests differed widely. Column A refers to an early test (Fig. 5) in which neither pteroylglutamic acid nor B_T was present; column B to a test (Figs. 7 and

8) in which liver-charcoal filtrate was added, as a source of B_T; column C to diets that contained purified B_T extract; and column D to larvae (Table 5) which had been grown on flour for an initial period of 4 weeks. Besides, columns A and B were conducted at 25° C. and columns C and D at 30° C., the higher temperature having the effect of speeding up development considerably. In spite of these diversities of conditions, several facts stand out clearly in these tests. The most striking feature is the fact that the larvae always died much sooner in the absence of riboflavin or pantothenic acid than when the other vitamins were lacking. Next in importance for survival is pyridoxin. The larvae survived still longer in the absence of thiamin or nicotinic acid, and there was no mortality at all in the absence of choline, though the growth rate was sometimes reduced.

In tests on the effect of biotin, the insoluble yeast fraction was omitted from the diet and was replaced by pure biotin. In all other respects the diets were iden-

TABLE 4

EXPERIMENT 416: *Tenebrio molitor*, GROWTH OF LARVAE IN THE PRESENCE OF GRADED DOSES OF THIAMIN, RIBOFLAVIN, NICOTINIC ACID, PYRIDOXIN, PANTOTHENIC ACID, OR CHOLINE*

Doses expressed in micrograms per gram of the diet. Average weight of larvae after 7 weeks (in mg.) and number of survivors out of 40. Minimum optimal quantities are italicized. At 30° C.

Thiamin			Riboflavin			Nicotinic Acid			Pyridoxin			Pantothenic Acid			Choline		
μgm.	Mg.	No.	μgm.	Mg.	No.	μgm.	Mg.	No.	μgm.	Mg.	No.	μgm.	Mg.	No.	μgm.	Mg.	No.
.....	2.2	34	.....	1	11	.....	1	34	.....	1.25	12	.....	.....	0	.....	20.6	36
0.25	13.4	35	0.5	1.4	13	2	3	35	0.25	4.6	31	1	1.2	4	11	25.3	36
0.5	25.9	39	1	2.1	23	4	8.3	33	0.5	18.9	38	2	1.7	10	33	20.5	38
1	*35.2*	*38*	2	9.4	19	8	24	39	1	22.4	37	4	12.1	20	100	25.7	37
2	36.7	39	4	23.0	38	*16*	*34.3*	*33*	*2*	*34.4*	*39*	8	*28.2*	*35*	*300*	*36.0*	*38*
4	36.2	38	8	*34.2*	*38*	32	34.5	35	4	32.5	36	16	29.7	36	900	35.5	34

* Controls: All vitamins present: 36 mg., 39 larvae.

TABLE 5

EXPERIMENT 415: GROWTH AND SURVIVAL OF *Tenebrio* LARVAE ON VARIOUS DEFICIENT DIETS

After larvae had been grown for the first 4 weeks on an optimal diet—whole-wheat flour plus 5 per cent yeast. Average weight of larvae after 4 weeks, 3.8 mg. Average weight in milligrams and number surviving out of 20, in parentheses, after 6–14 weeks at 30° C. and 70 per cent R.H.

Vitamin Omitted	Age of Larvae (in Weeks)								
	6	7	8	9	10	11	12	13	14
Thiamin	16.6 (20)	20.5 (20)	23.7 (20)	.....	.....	30.2 (20)	.....	.....	.....
Riboflavin	10.1 (20)	11.0 (18)	12.0 (15)	.....	.....	11.0 (8)	10.7 (7)	.....	.....
Nicotinic acid	13.3 (20)	16.0 (20)	16.5 (20)	.....	.....	17.0 (19)	16.1 (18)	.....	.....
Pyridoxin	14.9 (20)	17.7 (20)	19.0 (20)	.....	.....	20.1 (19)	20.3 (19)	.....	.....
Pantothenic acid	10.6 (20)	12.5 (20)	13.0 (16)	.....	.....	14.1 (11)	13.8 (10)	.....	.....
Choline chloride	21.2 (19)	33.0 (19)	52.5 (19)	70.1 (19)	85.9 (19)	95.5 (17)	.....	.....	.....
Biotin	14.7 (20)	21.7 (20)	27.5 (20)	37.3 (20)	42.6 (20)	49.1 (20)	56.1 (20)	64.0 (20)	68.4 (20)
Pteroylglutamic acid	20.3 (20)	32.5 (20)	43.0 (20)	52.1 (20)	55.4 (20)	56.8 (18)	58.6 (17)	.....	.....
None	20.3 (20)	34.1 (20)	52.5 (20)	75.1 (20)	91.3 (19)	.....	.....	.....	.....

tical with those used in testing for the other members of the B complex and contained a liver- or yeast-charcoal filtrate as a source of B_T. Figure 9 refers to a series of tests which were somewhat deficient in B_T and in which from one-third to one-half the larvae died by the twelfth week. Nevertheless, the effect of biotin, in quantities of 0.16 μgm/gm was very striking and approached that of the insoluble yeast fraction. Subsequently, an experiment was designed in which the effect of graded doses of biotin was investigated at two different temperatures, 25° and 30° C. (Table 7). Biotin was added in quantities of 0.08, 0.16, and 0.33 μgm/gm of the dry diet. Each test was performed with yeast-charcoal filtrate or liver-charcoal filtrate as sources of B_T in minimal optimal doses. The following results are worth noting:

1. The larvae reacted to graded doses of biotin with a graded response, those receiving the larger doses attaining the greatest weight.

2. An optimal quantity seemed to be 0.33 μgm/gm, with 0.16 μgm. approaching the same effect very closely. Even 0.08 μgm. always gave a very striking reaction.

TABLE 6

THE MORTALITY OF *Tenebrio molitor* LARVAE ON DIETS LACKING IN A SINGLE VITAMIN*

No. of Weeks	All Vitamins				No Thiamin				No Riboflavin				No Nicotinic Acid				No Pyridoxin				No Pantothenic Acid				No Choline			
	A†	B‡	C§	D‖	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
4	36	39	39	....	36	38	39	...	22	8	22	...	34	39	37	...	29	38	27	...	16	10	1	...	38	38	37	..
5	36	39	39	....	35	38	39	...	17	5	18	...	32	39	37	...	20	35	24	...	13	10	1	...	37	38	37	..
6	20	38	39	20	35	37	39	20	15	3	15	20	32	39	36	20	17	33	20	20	11	10	..	20	37	38	37	19
7	11	37	39	20	35	37	34	20	12	3	11	18	32	39	34	20	17	30	12	20	5	10	..	20	36	38	37	19
8	8	33	39	20	35	35	29	20	9	2	9	15	32	37	29	20	11	30	8	20	5	9	..	16	34	38	37	19
9	5	32	39	18	35	32	26	20	7	2	7	...	29	30	23	...	10	27	6	...	2	7	....		22	36	37	19
10	4	29	...	18	34	30	..	20	6	2	....		27	29	....		8	22	....		....	7	....		15	36	..	17
11	...	27	....	....	34	28	..	20	6	2	..	8	25	....	..	19	8	....	..	19	...	7	..	11	8	36	..	17
12	...	...	....	....	34	27	....		4	2	..	7	11	26	..	18	7	14	..	19	...	7	..	10	5	36	....	
13	...	...	....	....	29#	....	....	...	2	....	....	...	5	....	....	...	5	....	....	...	....	....	....	...	....	....	....	...

* Numbers of survivors are given out of 40 (cols. A, B, C) or 20 (col. D) larvae after 4–13 weeks.

† Experiment 218. The diets contain no source of B_T, at 25° C.

‡ Experiment 337. With liver-charcoal filtrate, at 25° C.

§ Experiment 416. With a B_T concentrate, at 30° C.

‖ Experiment 415. With a B_T concentrate, at 30° C. The larvae were grown for the first 4 weeks on whole meal plus 5 per cent yeast, and then transferred to experimental diets.

Eleven survivors only, after 16 weeks. Continued to die rapidly.

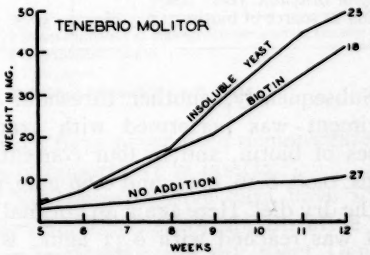


FIG. 9.—Growth of *Tenebrio* larvae in the presence and absence of biotin or insoluble yeast fraction. The diet contains a yeast-charcoal filtrate as a source of B_T. Numbers of survivors, out of 40, are given after 12 weeks. Temperature 25° C., relative humidity 70 per cent.

3. There is no indication that the yeast or liver extracts could have contained any significant amounts of biotin.

4. Raising the temperature of the tests from 25° to 30° speeded up develop-

ment very considerably and, after 10 weeks, nearly doubled the final weight. There was, however, no difference in quantitative requirements at the different temperatures.

5. In all the tests, except at 25° with yeast extract, growth was almost as good in the presence of biotin as with insoluble yeast fraction. This would suggest that the insoluble yeast fraction does not supply any unknown growth factors of importance for *Tenebrio*.

TABLE 7

EXPERIMENT 383: THE BIOTIN REQUIREMENTS OF *Tenebrio molitor*

Requirements determined in the presence of yeast-charcoal filtrate or liver-charcoal filtrate as sources of B_T, and at 25° and 30° C. Average weights after 10 weeks, and numbers of larvae out of 20.

Additions of Biotin	At 25° C.				At 30° C.			
	Yeast-Charcoal Filtrate		Liver-Charcoal Filtrate		Yeast-Charcoal Filtrate		Liver-Charcoal Filtrate	
	No.	Mg.	No.	Mg.	No.	Mg.	No.	Mg.
None	18	10.5	19	12	15	20.2	20	22.5
0.08 μgm/gm	16	22	18	22.7	18	68.8	19	63.1
0.16 μgm/gm	15	23	19	39	19	79	19	93.2
0.33 μgm/gm	15	34.6	19	42.3	19	89	20	90
2½% of insoluble yeast fraction as source of biotin	18	50.5	20	48.5	19	105	19	98

Subsequently, another threshold experiment was performed with graded doses of biotin, and in four concentrations, 0.08, 0.16, 0.33, and 0.66 μgm/gm of the dry diet. Here again an optimal effect was reached with 0.33 μgm., with 0.16 μgm. closely approaching the optimal and no further improvement with 0.66 μgm.

DISCUSSION

The food requirements of *T. molitor* closely resemble those of *Tribolium confusum*, *Ephestia kuehniella*, *Tineola biselliella*, *Dermestes vulpinus*, and other insects living in stored cereal products which we have previously investigated (Fraenkel and Blewett, 1943*a–d*; 1944*a*, *b*; 1946*a*, *b*). Its carbohydrate requirements are very high, being 80 per cent or more of the diet. In this respect *Tenebrio* resembles other insects like *E. kuehniella* or *Sitodrepa panicea*, which, under normal circumstances, also occur exclusively in cereal products. In fact, *Tenebrio* still develops nearly optimally with close to 90 per cent glucose in the diet and only 10 per cent of protein in the form of casein. Like other insects whose nutritional requirements have been investigated to date in detail, including *Drosophila* and mosquito larvae, *Tenebrio* requires a sterol, with cholesterol serving the purpose, and a full set of the known vitamins of the B complex, with the exception of inositol and para-aminobenzoic acid. *Tenebrio* differs from other insects in that it requires an additional factor, contained in yeast or liver, which has been provisionally named B_T (Fraenkel, Blewett, and Coles, 1948). We have succeeded in devising an artificial diet for *Tenebrio* which is composed of casein (20), glucose (80), cholesterol (1), salt mixture (2), and eight vitamins of the B complex. On such a diet, with the addition of a purified B_T concentrate in quantities of 3 μgm. solids

per gram of the diet, growth proceeds almost as fast as on the best natural diet, which is whole-meal flour plus yeast. Apart from one component, B_T, the isolation of which can be expected shortly, the problem of a "synthetic" diet for *Tenebrio* has been solved. It is true that the conditions of growth are not aseptic and that the presence of microörganisms might, conceivably, influence the nutritional requirements. Such microörganisms could occur only in the intestinal tract because the diet, in equilibrium with 70 per cent relative humidity, contains only 10 per cent of water and, for all practical purposes, is an unsuitable medium for the growth of microörganisms. The possibility of a nutritional effect of intestinal organisms seems, however, very remote in view of the strict requirements of *Tenebrio* for nine vitamins of the B complex. Six of these—thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, and B_T—are absolutely limiting factors, in that growth ceases entirely in their absence. Only in the case of biotin, pteroylglutamic acid, and choline is there a theoretical possibility of limited intestinal synthesis. In the absence of biotin and pteroylglutamic acid, very slow growth is possible, and, in the absence of choline, growth is moderate.

In its qualitative requirements of the members of the vitamin B complex, *Tenebrio* closely resembles most other insects which have been investigated with reference to the effect of these vitamins on growth. It appears that the need of insects for the eight vitamins mentioned above is of general occurrence. Apparent exceptions to this rule have been explained as due to insufficient purity of some of the ingredients of the diet, e.g., the casein in the case of folic acid (Fraenkel and Blewett, 1947), or to the presence and synthetic action of intra- or extra-cellular microörganisms (Fraenkel and Blewett, 1944*a*; Brecher and Wiglesworth, 1944). The presence of intracellular microörganisms may also offer an explanation for the rather aberrant vitamin requirements of the roach, *Blatella germanica*, in which complete omission of choline from the diet results in a complete cessation of growth, while omission of each of the other vitamins singly results in less marked effect upon growth and survival (Noland, Lilly, and Baumann, 1949). Inositol and para-aminobenzoic acid are the only chemically known components of the B complex which have never been demonstrated to be of importance in insects.

TABLE 8

MINIMAL OPTIMUM DOSES OF VITAMIN REQUIREMENTS FOR TWO INSECTS, *Tribolium confusum* AND *Tenebrio molitor*, AND FOR CHICKS*

Vitamin	*Tribolium*†	*Tenebrio*‡	Chicks§
Thiamin	1	1	2
Riboflavin	1–2	2–8	3.6
Nicotinic acid	8	16	18
Pyridoxin	1	2	3.6
Pantothenic acid	4	8	11.2
Choline	.....	300	1,600
Biotin	0.05	0.16	0.1
Pteroylglutamic acid	0.125–0.25	0.125	0.6

* Doses expressed as micrograms per gram of the dry diet.
† Unpublished results.
‡ This paper.
§ National Research Council, 1945.

No comprehensive data have so far been published on the quantitative vitamin requirements of any insect. Generally speaking, the requirements of *Tenebrio* for eight vitamins, as stated in the preceding pages, and for pteroylglutamic acid (Fraenkel and Blewett, 1947) closely resemble those of *T. confusum* (Table 8). They are also strikingly similar to corresponding data concerning the chick (National Research Council, 1945; Johnson, 1948), though these data must be assumed to have been chosen somewhat too

high. It is well known that minimal optimal requirements of vitamins cannot be stated in absolute terms, as they usually vary in the best-controlled experiments by a factor of 100 per cent and more. In our own tests, for instance, the requirements of *Tenebrio* for riboflavin were found to be 2.0 μgm/gm on one occasion and 8.0 on another. For these reasons it is not intended to draw any conclusions from apparent differences, as between species, in the data presented in Table 8, except for choline and riboflavin. There seems to be a significant difference between *Tenebrio* and *Tribolium* in the riboflavin requirements. In our experience there was never an indication that whole-meal flour was not an optimal diet for *Tribolium*, *Ephestia*, or other flour insects, while *Tenebrio* grows very slowly on whole meal alone, as has been reported in several instances before (Lafon and Teissier, 1939; Martin and Hare, 1942; Leclercq, 1948*b*). We have now demonstrated that this deficiency is principally due to a lack of riboflavin in whole meal. This fact goes far to explain the distribution of *Tenebrio* in flour mills, granaries, and elevators, where it never infests the stored grain or flour but rather occurs in the mixture of odd-grain remnants in corners or below the floor, which largely represent fragments of bran or seed coat and have a high riboflavin content. *Tenebrio* differs in this respect from *T. confusum* and *E. kuehniella*, which are less susceptible to a riboflavin shortage and are true pests of flour. But even these species grow relatively slowly on white flour, which has a very low riboflavin content; and we have previously demonstrated (Fraenkel and Blewett, 1941) that the addition of riboflavin renders white flour a more suitable food for *T. confusum*.

There seem also to be significant differences in the choline requirements of different insects. Results pertaining to the effect of omission of choline from the diet of *Tribolium* and *Tenebrio* were not always consistent, but both insects have afforded cases of a clear-cut choline deficiency. On one such occasion 300 μgm. of choline per gram gave an optimal effect, but the actual requirements in this test (Table 4) must have been considerably lower. These amounts are only a small fraction of the requirements of chicks (1,600 μgm.) and the German roach (2,000–4,000 μgm., Noland and Baumann, 1949). It would appear that both *Tribolium* and *Tenebrio* possess limited powers of choline synthesis.

The food requirements of *T. molitor* have been the subject of three previous investigations. The work of Lafon and Teissier (1939), which was of only preliminary nature, established the need for large proportions of carbohydrates in the diet and the importance of yeast as an addition to white flour and dealt with the relative efficiency of different carbohydrates. Martin and Hare's (1942) synthetic diet made possible only very slow growth. Nevertheless, the need for thiamin, riboflavin, nicotinic acid, pyridoxin, and pantothenic acid was clearly demonstrated. The diet was not improved by the addition of vitamins A, C, D, E, K, or choline. We know now that this diet must have been lacking in biotin, pteroylglutamic acid, and B_T. Even so, growth in the absence of these three factors could never have been so successful as stated by the authors if the larvae had been grown from the beginning on the synthetic diet, instead of being maintained for several initial weeks on a complete natural diet. There is also the possibility that the starch and casein might have contained important growth factors.

Recently Leclercq (1948*b*) reported results of nutritional experiments with *Tenebrio*, using the diet previously de-

veloped by us. Although hardly any details about the composition of the diet and the results are given, it appears that the importance of cholesterol and of thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, and "folic acid" was recognized. Growth on the "complete" diet was, however, very slow and was only slightly improved by the substitution of starch for glucose. It appears that these diets must have been deficient in choline, biotin, and B_T. The author also stresses the fact that larvae which had been grown on a natural diet up to half- or nearly full size may dispense with additional factors which are necessary for newly hatched larvae. This observation is easily explained as an effect of vitamin storage in the tissues, and we made use of this phenomenon in our early tests when, in the absence of pteroylglutamic acid and B_T, the larvae failed to grow satisfactorily on synthetic diets and died at an early stage.

In our experience, *Tenebrio*, by comparison with *Tribolium*, *Ephestia*, and other insects of stored products, offers many advantages for nutritional research. Like other insects of this type, it grows well on a powdery medium with a water content of only 10 per cent or even less. Its much larger size makes it easy to handle, either singly or in groups, and to measure growth by weighing at regular intervals. Thus experimental results may be obtained in only a few weeks and long before the insects are fully grown. Weight increase is a quicker and more accurate criterion for measuring the efficiency of diets than is the length of the larval period, which was the standard method used in our previous work with *Tribolium*, *Ephestia*, and other insects. It eliminates the prepupal period, which, in many insects, shows considerable individual variation in length. *Tenebrio* larvae reach a size of 140–80 mg., which is large enough to make dissection methods possible. Finally, *Tenebrio*, like many other insects of stored products can be conveniently bred all the year round in the laboratory, and eggs or larvae of a required age can be obtained with the greatest of ease at the required time and in the required numbers.

SUMMARY

1. Details are given about the composition of artificial diets for the mealworm, *T. molitor*, and the methods of testing for nutritional deficiencies.

2. The rate of growth of *Tenebrio* very much depends on the water content of the food, growth being fastest on food in equi'ibrium with 70 per cent R.H., very slow at 30 per cent R.H., and hardly proceeding at 13 per cent R.H.

3. *Tenebrio* requires a high percentage, 80–85 per cent, of carbohydrate in the diet. The effects of glucose and starch are similar.

4. Data are given on growth in the presence of different proteins. Casein, lactalbumin, and peanut protein seem to give optimal effects; soybean protein becomes satisfactory only after extensive heating, while zein, gliadin, and enzymatic hydrolyzates from casein or lactalbumin are of only slight value.

5. Cholesterol is a necessary ingredient of the diet. There is no indication that fat is required.

6. Whole-wheat flour is of only slight nutritional value but becomes an optimal food with the addition of 5 per cent yeast. The deficiency is principally due to a lack in riboflavin.

7. *Tenebrio* requires the following vitamins of the B complex, minimal amounts to produce an optimal response being given in parentheses expressed as micrograms per gram of the dry diet: thiamin (1), riboflavin (2–8), nicotinic acid (16),

pyridoxin (2), pantothenic acid (8), biotin (0.16), pteroylglutamic acid (0.12), and choline (300). In the absence of choline, growth is somewhat depressed. *Tenebrio* also requires a yet undetermined factor, B_T.

8. No growth whatsoever takes place in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, or pantothenic acid; very slow growth is possible without biotin or pteroylglutamic acid, but growth is little affected without choline; there is no indication that inositol or para-aminobenzoic acid are required.

9. In the absence of riboflavin or pantothenic acid, the larvae die much sooner than in cases of deficiencies of the other B vitamins.

LITERATURE CITED

BRECHER, G., and WIGGLESWORTH, V. B. 1944. The transmission of *Actinomyces rhodnii* Erikson in *Rhodnius prolixus* Stal (Hemiptera) and its influence on the growth of the host. Parasitology, 35:220–24.

EL SADR, M. M.; MACRAE, T. F.; and WORK, C. E. 1940. The estimation of riboflavin. Biochem. Jour., 34:600–612.

FRAENKEL, G. 1948. The effects of a relative deficiency of lysine and tryptophane in the diet of an insect, *Tribolium confusum*. Biochem. Jour., Proceedings, 43:xiv.

———. 1950. The isolation of vitamin B_T (unpublished).

FRAENKEL, G., and BLEWETT, M. 1941. Deficiency of white flour in riboflavin (tested with the flour beetle *Tribolium confusum*). Nature, 147:716.

———. 1943*a*. The basic food requirements of several insects. Jour. Exper. Biol., 20:28–34.

———. 1943*b*. The vitamin B-complex requirements of several insects. Biochem. Jour., 37:686–92.

———. 1943*c*. The sterol requirements of several insects. *Ibid.*, pp. 692–95.

———. 1943*d*. The natural foods and the food requirements of several species of stored products insects. Trans. Roy. Ent. Soc. London, 93: 457–90.

———. 1944*a*. Intracellular symbiosis and vitamin requirements of two insects, *Lasioderma serricorne* and *Sitodrepa panicea*. Proc. Roy. Soc. London, B, 132:212–21.

———. 1944*b*. The utilization of metabolic water in insects. Bull. Ent. Res., 35:127–39.

———. 1946*a*. The dietetics of the clothes moth, *Tineola biselliella* Hum. Jour. Exper. Biol., 22:156–61.

———. 1946*b*. The dietetics of the caterpillars of three *Ephestia* species, *E. kuehniella*, *E. elutella* and *E. cautella*, and of a closely related species, *Plodia interpunctella*. *Ibid.*, pp. 162–71.

———. 1946*c*. Linoleic acid, vitamin E and other fat-soluble substances in the nutrition of certain insects (*Ephestia kuehniella*, *E. elutella*, *E. cautella* and *Plodia interpunctella*). *Ibid.*, pp. 172–90.

———. 1947. The importance of folic acid and unidentified members of the vitamin B complex in the nutrition of certain insects. Biochem. Jour., 41:469–75.

FRAENKEL, G.; BLEWETT, M.; and COLES, M. 1948. B_T, a new vitamin of the B-group and its relation to the folic acid group and other anti-anaemia factors. Nature, 161:981–83.

FRAENKEL, G., and CHAYEN, J. Studies on the utilization of the food in the larva of *Tenebrio molitor* (unpublished).

JOHNSON, B. C. 1948. New developments in livestock feeding—some recent research in animal nutrition. Rapp. VII[e] Cong. Internat. Ind. Agr. Q9, pp. 12–18. Paris: Juillet, 1948.

KUNITZ, M. 1947. Crystalline soybean trypsin inhibitor. II. General properties. Jour. Gen. Physiol., 30:291–310.

LAFON, M., and TEISSIER, G. 1939. Les besoins nutritifs de la larve de *Tenebrio molitor*. Compt. rend. Soc. biol., 131:75–77.

LECLERCQ, J. 1948*a*. Importance de la lysine et du tryptophane dans la nutrition de *Tenebrio molitor* L. Experientia, 4:436.

———. 1948*b*. Sur les besoins nutritifs de la larve de *Tenebrio molitor* L. Biochim. et biophys. acta, 2:329–32.

———. 1948*c*. Contribution à l'étude du métabolisme de l'eau chez la larve de *Tenebrio molitor* L. Arch. internat. physiol., 55:412–19.

———. 1948*d*. Importance de glucides dans la nutrition des larves de *Tenebrio molitor* L. *Ibid.*, 56:28–34.

MARTIN, H. E., and HARE, L. 1942. The nutritive requirements of *Tenebrio molitor* larvae. Biol. Bull., 83:428–37.

NATIONAL RESEARCH COUNCIL. 1945. Recommended nutrient allowances for poultry. Washington, D.C.: Government Printing Office.

NOLAND, J. L., and BAUMANN, C. A. 1949. Requirements of the German cockroach for choline and related compounds. Proc. Soc. Exper. Biol. and Med., 70:198–201.

NOLAND, J. L.; LILLY, J. H.; and BAUMANN, C. A. 1949. Vitamin requirements of the cockroach *Blatta germanica*. Ann. Ent. Soc. Amer., 42: 154–64.

THE CONCENTRATION OF CERTAIN INORGANIC CONSTITUENTS IN THE BLOOD OF THE CYNTHIA PUPA SAMIA WALKERI FELDER AND FELDER[1]

PHYLLIS KRING GESE
Department of Biology, University College, New York University

THERE is a dearth of information concerning the concentration of the constituents of insect blood. Relatively few studies have been made on this subject, and most of them are limited in scope. The present report is limited to the concentration of chlorides, potassium, sodium, calcium, magnesium, and inorganic phosphate in the blood of the Cynthia pupa.

MATERIALS AND METHODS

Cynthia pupae, in cocoons, were collected in New York City in the spring and fall of 1948. The pupae, in their cocoons, were maintained in the laboratory at a temperature of approximately 5° C. to prevent emergence. When an analysis was to be made, the pupae were removed from their cocoons, and blood was obtained from each pupa by puncturing with a glass needle the body wall at the base of each antenna. For each determination the blood (from a sufficient number of pupae to fulfil procedure requirements) was collected by being allowed to drip freely into a centrifuge tube. From this pooled blood samples were taken by pipette for individual analyses.

All glassware used in the present work was washed thoroughly with a detergent and then soaked overnight in strong chromic-sulfuric cleaning solution. This was followed by several rinses with cold distilled water, two rinses with hot distilled water, and two rinses with hot, Pyrex redistilled water. All flasks and centrifuge tubes were steamed over a permanganate bath as a final rinse and were drained dry in an inverted position.

All chemicals used were either C.P. or reagent grade. All solutions were prepared with Pyrex redistilled water and stored in chemically clean bottles. In each determination employing a titrimetric procedure all titrating solutions were standardized by accepted methods. It was found that the methods used for determining the amount of certain constituents in mammalian blood are quite satisfactory for the analysis of insect blood, if slight modifications are made. In cases in which great differences exist in the concentration of a particular constituent in mammalian and insect blood, the quantity of the blood sample was changed to qualify it for the technique. Calculations were modified accordingly.

The chlorides were determined by the micromethod of Van Slyke (1923). The titration with standard sulfocyanate was done according to the Whitehorn (1921) modification of the Volhard titration. The concentration of chlorides was calculated from the corrected amount of 0.02 N thiocyanate solution required in

[1] Submitted in partial fulfilment of the requirements for the degree of Master of Science at New York University. The author wishes to express appreciation to Professor Jean Leclercq, Department of Biochemistry, University of Liége, for suggesting the problem; to Dr. Daniel Ludwig, for his advice about and criticism of the manuscript; and to Dr. William Cosgrove, for many invaluable suggestions pertaining to the technical phases of the work.

the titration; the concentration of sodium chloride was also calculated from this value.

The amount of potassium in the blood was determined according to the method of Shohl and Bennett (1928), as modified by Fenn and Cobb (1935), and as further modified by Wilde (1939). The titration of each sample was done with a microburette of 2-ml. capacity, graduated to 0.01 ml. Because the blood of the insect contains such large amounts of potassium, the strength of the sodium thiosulfate was increased from 0.0025 N to 0.01 N, the exact potassium equivalent of which was determined by the analysis of a standard potassium solution.

The determination of sodium was done by the micromethod devised by Weinbach (1935), which is based on a modification of the colorimetric procedure of Salit (1932); the latter method is a modification of the uranyl–zinc acetate gravimetric procedure devised by Barber and Kolthoff (1929). Because of the small amount of sodium in insect blood it was found necessary to use 1 ml. of blood diluted to a volume of 2 ml. (by the addition of 0.6 ml. of water and 0.4 ml. of trichloroacetic acid) instead of 0.1 ml. of blood required by the original procedure. Additional amounts of trichloroacetic acid did not cause further protein precipitation in the controls tested. The sodium hydroxide, used in the titration, was standardized with a sodium chloride solution of known concentration. Following a suggestion given by Hawk, Oser, and Summerson (1947), carbon dioxide–free water was used to dissolve the precipitate (after the final washing), so that the titration would not be affected by carbonic acid.

The calcium concentration was determined by the method of Clark and Collip (1925), which is a modification of the Tisdall method (1923) for the determination of calcium in small amounts of serum. Sendroy (1944) showed that this method gives accurate results and that the preliminary removal of protein is not required. In addition to being an accurate and simple technique, the supernatant liquid can be used for the colorimetric determination of magnesium. This procedure, therefore, saves time and materials. The calcium-concentration values were calculated from the amount of permanganate required for the titration.

The determination of magnesium, from the supernatant liquid of the calcium determination, was done according to the method of Denis (1922) as modified by Fiske and Subbarow (1925). In the precipitation of magnesium, three additional drops of ammonium hydroxide were added to produce larger crystals. It was also imperative to use centrifuge speeds of 1,700–2,000 rpm to pack the very light precipitate firmly enough to avoid any loss during the washing process. Dilution of the supernatant liquid, from the calcium determination, was necessary because of its high magnesium content. Accurate readings were possible when a 1:25 dilution was made. Dilution of the blood before the removal of the calcium resulted in too great a variation of the final values for magnesium. A Coleman Junior spectrophotometer was used in the determination to eliminate the visual errors usually found when a colorimeter is used.

The method of Fiske and Subbarow (1925) was used for the determination of inorganic phosphate. The colorimetric determination of phosphate was originally developed by Bell and Doisy (1920) and modified by Briggs (1922). Phosphate values, reported in the literature for other insects are comparatively large. It was, therefore, necessary to make adequate dilutions of the insect blood, so that the concentration of the phosphate

would approximate the value for human blood. By making a 1:21 dilution, it was possible to obtain accurate readings, using a photometer. The phosphate concentration was determined photometrically, using a simultaneously prepared standard.

EXPERIMENTAL RESULTS

The results of the various determinations are expressed in the tables in terms of both milliequivalents per liter (except phosphorus) and milligrams per cent. Expressing the molecular concentration as milliequivalents per liter facilitates the use of these values in possible studies of the acid-base balance of the blood.

Table 1 contains values expressing the concentration of the chloride ion in the blood of the Cynthia pupa. The concentration of chlorides has also been given in terms of mg. per cent NaCl, in accordance with the practice of other workers in this field.

Tables 2–5 list the concentration values of potassium, sodium, calcium, and magnesium, respectively. Potassium values are very high, and sodium values are very low. The relative concentration of all four of these ions agrees with the results of other studies of insect blood (see Wigglesworth, 1939, p. 230).

The phosphorus concentration (Table 6) is not expressed in terms of milliequivalents per liter because no pH measurements were made, and, therefore, the ratio $[H_2PO_4^-]/[HPO_4^{--}]$ is unknown.

TABLE 1

CHLORIDE CONCENTRATION

Sample	Milliequivalents Cl^-/Liter	Mg. Per Cent Cl^-	Mg Per Cent NaCl
1	10.87	38.60	63.59
2	10.87	38.60	63.59
3	10.87	38.60	63.59
4	9.81	34.84	57.39
5	9.81	34.84	57.39
6	9.81	34.84	57.39
7	10.87	38.60	63.59
Average	10.416	36.989	60.936

TABLE 2

POTASSIUM CONCENTRATION

Sample	Milliequivalents K^+/Liter	Mg. Per Cent K^+
1	40.93	160.0
2	43.20	168.9
3	42.87	167.6
4	41.18	161.0
5	40.34	157.7
6	44.05	172.2
Average	42.095	164.6

TABLE 3

SODIUM CONCENTRATION

Sample	Milliequivalents Na^+/Liter	Mg. Per Cent Na^+
1	2.02	4.64
2	2.02	4.64
3	2.44	5.60
4	2.13	4.90
5	3.03	6.96
6	2.67	6.15
7	3.19	7.34
8	2.80	6.44
9	2.82	6.49
10	2.86	6.57
Average	2.597	5.973

TABLE 4

CALCIUM CONCENTRATION

Sample	Milliequivalents Ca^{++}/Liter	Mg. Per Cent Ca^{++}
1	18.81	37.7
2	18.86	37.8
3	19.12	38.3
4	18.91	37.9
5	18.76	37.6
6	18.81	37.7
7	19.06	38.2
8	18.81	37.7
9	18.21	36.5
Average	18.815	37.71

The concentration of any blood constituent in insects of the same species, living under similar conditions, might be expected to differ only slightly. In the present study the blood of a number of Cynthia pupae, living under similar conditions, was pooled. Consistent results were obtained for each analysis, as might be expected.

TABLE 5

MAGNESIUM CONCENTRATION

Sample	Milliequivalents Mg^{++}/Liter	Mg. Per Cent Mg^{++}
1	65.07	79.13
2	65.69	79.88
3	65.21	79.30
4	64.46	78.38
5	64.46	78.38
6	66.03	80.30
7	64.25	78.13
8	65.07	79.13
Average	65.03	79.08

The studies of Bishop, Briggs, and Ronzoni (1925) on the blood of the honey-bee larva included a complete analysis of the blood and the determination of osmotic-pressure values. They failed, however, in their attempt to make an adequate physiological salt solution for the insect. Nevertheless, they discovered the important fact that "osmotically the amino acids are the most effective fraction, having twice the value of the inorganic salts." Osmotic-pressure calculations cannot be made from the values determined in this study, because only a portion of the blood components was determined.

Too many variables are involved to attempt any correlation between the concentration values found in this study with those obtained for other insects. This is especially true in view of the relatively insignificant amount of information available for purposes of comparison. When a greater variety of insects has been studied, differences in the concentration of blood constituents may possibly be correlated with differences in dietary habits, which in turn are related to stages of metamorphosis, types of mouth parts, and the specific type of food available.

TABLE 6

INORGANIC PHOSPHATE CONCENTRATION

Sample	Mg. Per Cent P
1	10.95
2	10.95
3	11.15
4	10.95
5	11.05
6	11.05
7	11.05
8	11.05
9	10.95
Average	11.015

SUMMARY

1. The blood of pupae of the Cynthia moth, *Samia walkeri*, was quantitatively analyzed for certain of its inorganic constituents.

2. The average concentration values are: chlorides, 36.99 mg. per cent; sodium, 5.97 mg. per cent; potassium, 164.6 mg. per cent; calcium, 37.71 mg. per cent; magnesium, 79.08 mg. per cent; and inorganic phosphate, 11.015 mg. per cent.

LITERATURE CITED

BARBER, H. H., and KOLTHOFF, I. M. 1929. Gravimetric determination of sodium by the uranyl zinc acetate method. II. Application in the presence of rubidium, cesium, potassium, lithium, phosphate, or arsenate. Jour. Amer. Chem. Soc., 51:3233–37.

BELL, R. D., and DOISY, E. A. 1920. Rapid colorimetric methods for the determination of phos-

phorus in urine and blood. Jour. Biol. Chem., 44:55–67.

BISHOP, G. H.; BRIGGS, A. P.; and RONZONI, E. 1925. Body fluids of the honey bee larva. II. Chemical constituents of the blood, and their osmotic effects. Jour. Biol. Chem., 66:77–88.

BRIGGS, A. P. 1922. A modification of the Bell-Doisy phosphate method. Jour. Biol. Chem., 53:13–16.

CLARK, E. P., and COLLIP, J. B. 1925. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. Jour. Biol. Chem., 63:461–64.

DENIS, W. 1922. The determination of magnesium in the blood, plasma, and serum. Jour. Biol. Chem., 52:411–15.

FENN, W. O., and COBB, D. M. 1935. Evidence for a potassium shift from plasma to muscles in response to an increased carbon dioxide tension. Amer. Jour. Physiol., 112:41–55.

FISKE, C. H., and SUBBAROW, Y. 1925. The colorimetric determination of phosphorus. Jour. Biol. Chem., 66:375–400.

HAWK, P. B.; OSER, B. L.; and SUMMERSON, W. H. 1947. Practical physiological chemistry. Philadelphia: Blakiston Co.

SALIT, P. W. 1932. A new triple acetate method for sodium determinations in biological materials. Jour. Biol. Chem., 96:659–72.

SENDROY, J., JR. 1944. Determination of serum calcium by precipitation with oxalate. Jour. Biol. Chem., 152:539–56.

SHOHL, A. T., and BENNETT, H. B. 1928. A micro method for the determination of potassium iodoplatinate. Jour. Biol. Chem., 78:643–51.

TISDALL, F. F. 1923. A note on the Kramer-Tisdall method for the determination of calcium in small amounts of serum. Jour. Biol. Chem., 56: 439–41.

VAN SLYKE, D. D. 1923. The determination of chlorides in blood and tissues. Jour. Biol. Chem., 58:523–29.

WEINBACH, A. P. 1935. A micromethod for the determination of sodium. Jour. Biol. Chem., 110: 95–99.

WHITEHORN, J. C. 1921. A system of blood analysis. Supplement II. Simplified method for the determination of chlorides in blood or plasma. Jour. Biol. Chem., 45:449–60.

WIGGLESWORTH, V. B. 1939. The principles of insect physiology. New York: E. P. Dutton.

WILDE, W. S. 1939. The distribution of potassium in the cat after intravascular injection. Jour. Biol. Chem., 128:309–17.

COMPARISON OF BLOOD VOLUME IN ANIMALS WITH OPEN AND WITH CLOSED CIRCULATORY SYSTEMS

C. LADD PROSSER AND S. J. F. WEINSTEIN

Department of Physiology, University of Illinois, Urbana, Illinois

WHEN the dye T-1824 (Evans blue) is injected into the blood stream of mammals, it complexes with serum albumin and diffuses out of the capillaries very slowly; hence its observed dilution gives an approximate measure of the volume of circulating plasma. The salt NaCNS, conversely, leaves the capillaries but does not enter most tissue cells rapidly; hence its dilution is taken to represent the total extracellular volume. The T-1824 volume may be an inaccurate measure of plasma volume because mixing in blood depots may be incomplete. Measurements of "extracellular fluid volume" by different methods yield slightly different results; hence the thiocyanate volume is considered as an approximate, rather than a true, measure of extra-cellular volume.

In several laboratory mammals the blood volume calculated from T-1824 volume and hematocrit is equivalent to 7–8.5 per cent of body weight, and the thiocyanate volume varies from 25 to 35 per cent of body weight. The difference between the specific gravity of the blood and that of the whole body is less than differences in specific gravity of individual animals; hence in mammals the blood volume is essentially the same whether expressed as percentage of body weight or of body volume. Blood volumes calculated by dye dilution are similar to vol-

umes obtained by the older method of hemoglobin dilution, although there is much disagreement regarding accuracies; dilution of a radioactive protein may give more reliable measurements of blood volume (Krieger *et al.*, 1948). There are reports (Reichert and Brown, 1909; Derrickson and Amberson, 1934) that the blood volume of bony fish is much less than that of mammals. Among invertebrate animals no measurements of T-1824 volume have been made, and the only two reports of thiocyanate volume are in *Carcinus*, 37 per cent of body weight (Nagel, 1934), and in *Eriocheir*, 33 per cent (Krogh, 1939). The present investigation is a comparison of the T-1824 and thiocyanate volumes of the crayfish and fresh-water clam, animals with open circulation, with these volumes in the frog and catfish. If the partitioning of the dye and the thiocyanate are as postulated in mammals, both substances should measure the same volume in animals with open circulatory systems.

MATERIALS AND METHODS

The methods of Gregerson and Stewart (1939) were used with appropriate modifications. The dye (T-1824, Eastman Kodak Co.) was prepared at a concentration of 0.5 mg/ml. A dilution–optical-density curve was made by means of a Lumetron colorimeter with a color filter of wave-length 650 mμ; over the range used, one optical density unit was equivalent to 0.0191 mg. of T-1824. A stock solution of NaCNS was prepared and standardized at 0.2919 normal. For colorimetric assay the NaCNS was converted to $Fe(CNS)_3$ by means of $Fe(NO_3)_3$ acidified with HNO_3 and was read on the Lumetron colorimeter with a filter of 490 mμ. In most of the determinations small amounts of plasma were used and were diluted so much with water that absorption by the plasma could be disregarded, although checks were made with each type of blood. Some of the determinations were done with a Coleman spectrophotometer set at 620 mμ. for the dye and 480 mμ. for the thiocyanate.

The animals used were crayfish (*Cambarus virilis*), fresh-water mussels (mostly *Lampsilis ventricosa* and *Amblema costata*), frogs (*Rana pipiens* and *R. catesbiana*), and bullheads (*Ameiurus natalis*). Measured amounts of dye and thiocyanate solutions were injected into the heart, and small volumes of blood were periodically withdrawn in calibrated tuberculin syringes.

When either the dye or the thiocyanate was used separately, the method of handling the blood was simpler than when dye and thiocyanate were used together. The crayfish or clam blood was diluted ten to forty times with isotonic saline or distilled water; and, if the sample contained dye, the reading was made directly against a blank, or, if it contained NaCNS, a measured amount of $Fe(NO_3)_3$ was added and the optical density measured. Frog or fish blood was placed in a tube containing a few crystals of sodium citrate, it was centrifuged, the hematocrit measured, plasma diluted, and the optical density read colorimetrically. When crayfish or clam blood contained both dye and thiocyanate, one portion was diluted and read for T-1824, the remainder was diluted, protein was precipitated with trichloracetic acid to remove the dye, and the filtrate was used for thiocyanate measurement. The plasma of frog and fish blood was similarly treated, the dye-protein complex being precipitated before thiocyanate measurement. Results were calculated as follows:

$$\frac{\text{Mg. of T-1824 or NaCNS injected}}{\text{Mg. per ml. withdrawn}} = \text{T-1824 or NaCNS space in ml.}$$

The fluid volume was then expressed in percentage of body weight or body volume.

EXPERIMENTAL RESULTS

Crayfish (*C. virilis*).—Measurements were made with eighteen crayfish, ranging in weight from 10.5 to 48 gm. No consistent difference was noted with respect to size or sex. Measurement of T-1824 volume alone was made on seven animals, of NaCNS volume alone on seven, and of both simultaneously on four. Amounts injected were: 0.2 ml. containing 0.1 mg. of dye and 0.1 ml. containing 2.36 mg. of NaCNS; injections were made into the heart from the ventral aspect, entering just caudal to the third walking leg. Samples of 0.1 ml. were withdrawn from the abdominal sinus at 2, 5, 10, 15, and 30 minutes after injection. The cell volume was so small that no hematocrit correction was necessary. Time-concentration curves were made for each animal, and several typical curves are given in Figure 1. The T-1824 curves fell gradually and were readily extrapolated back to zero time to obtain the value of dye dilution.

Five of the time-concentration curves for thiocyanate were flat or showed gradual decrease in concentration, while in another five a gradual increase in concentration occurred, reaching a maximum between 10 and 15 minutes after injection. In these latter experiments the maximum concentration was taken for the volume measurement. The crayfish sometimes died in 20–30 minutes after injection of the NaCNS, while, after injection of the T-1824, they lived at least 24–48 hours. To investigate the thiocyanate effect, the hearts of five crayfish were exposed; and, when 0.1 ml. containing 2.36 mg. of NaCNS was injected directly into the heart, the beat was weak and spasmodic. When 0.2 ml., containing 0.1 mg. of T-1824, was injected, the heart beat remained regular and strong. When the thiocyanate was injected in 0.2 ml. of crayfish saline (Van Harreveld's solution) or in 0.2 ml. of dye solution, the heart continued to beat in a nearly normal manner. Apparently, in the amount and concentration needed for accurate determinations, NaCNS is toxic for the crayfish heart; hence mixing with the blood is slow, and the maximum concentrations obtained in blood from the abdominal sinus must be used in calculating the thiocyanate volume.

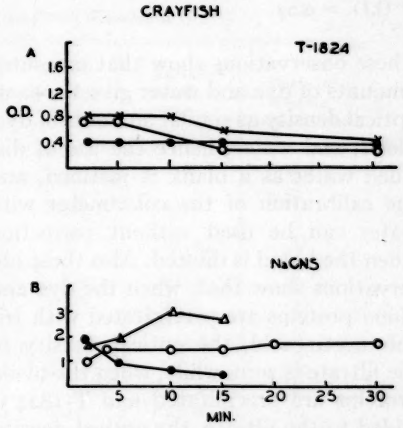


FIG. 1.—Typical time-concentration curves for crayfish. *O.D.*, relative optical density, as function of time in minutes after injection. *A*, selected curves for T-1824; *B*, curves for NaCNS.

Several *in vitro* tests were made with measured amounts of crayfish blood and distilled water, to learn whether the presence of blood in the colorimeter blank was essential to a valid reading and to discover whether T-1824 complexes with the protein constituents of the blood. Experimental data of one such test follow:

1. 1 ml. T-1824 (0.005 mg.)
   4 ml. distilled water
   Optical density (O.D.) = 0.05

2. 1 ml. T-1824
   0.1 ml. crayfish blood
   3.9 ml. distilled water
   O.D. = 0.05

3. 1 ml. T-1824
   0.1 ml. crayfish blood
   1 ml. CCl_3COOH. Let stand 10 minutes. Filter. To filtrate add distilled water to 5 ml.
   O.D. = 0.0

4. 0.1 ml. crayfish blood
   1 ml. CCl_3COOH. Let stand 10 min. Filter. To filtrate add 1 ml. T-1824 and water to 5 ml.
   O.D. = 0.05

These observations show that measured amounts of dye and water give the same optical density as similar amounts of dye, blood, and water; hence the use of distilled water as a blank is justified, and the calibration of the colorimeter with water can be used without correction when the blood is diluted. Also these observations show that, when the dye and blood proteins are precipitated with trichloracetic acid, the optical density of the filtrate is zero, while, when the blood proteins are precipitated and T-1824 is added to the filtrate, the optical density is the same as for dye plus water or dye plus blood and water. Hence the T-1824 complexes with proteins in crayfish blood, as it does in mammalian blood. Whether these proteins in the crayfish blood are albumins or other fractions is not known.

Similar *in vitro* tests were made with thiocyanate as follows:

1. 1 ml. NaCNS (0.0236 mg.)
   0.25 ml. $Fe(NO_3)_3$
   3.75 ml. distilled water
   O.D. = 0.217

2. 1 ml. NaCNS
   0.1 ml. crayfish blood
   0.25 ml. $Fe(NO_3)_3$
   3.65 ml. distilled water
   O.D. = 0.219

3. 0.1 ml. crayfish blood
   1.0 ml. NaCNS
   0.3 ml. CCl_3COOH. Remove precipitate by centrifugation.
   0.2 ml. $Fe(NO_3)_3$
   3.4 ml. water
   O.D. = 0.217

These experiments demonstrate that the optical density is essentially the same with crayfish blood present with water, NaCNS, and $Fe(NO_3)_3$ as when no blood is present. The reading was also the same as if the protein had been precipitated with trichloracetic acid; hence NaCNS does not complex with blood proteins.

Measurements of T-1824 volume and thiocyanate volume are given in Table 1. The dye volume ranged from 10 to 43 per cent, with a mean of 25.1 per cent of body weight. The NaCNS volume ranged from 19 to 48 per cent, with a mean of 25.6 per cent of body weight. The results obtained with simultaneous measurements agree well with the separate measurements of the two volumes. These results show that in the crayfish the T-1824 space is equivalent to the NaCNS space.

Unfortunately, body volume was not measured in the crayfish in which dye and thiocyanate volumes were measured. However, the specific gravity of five crayfish, including carapace, averaged 1.22 (limits 1.06–1.38); the specific gravity of crayfish blood is given as 1.018 (Ellis, Meirick, and Ellis, 1930). With these values and a blood volume equal to 25.1 per cent of body weight, the weight of the blood would be 25.5 per cent of body weight and the volume of the blood 30.5 per cent of the body volume.

Fresh-water mussels.—Two series of measurements were made on mussels, one series of twenty-two animals in the summer of 1948, the other of eleven animals in the fall of 1949. A hole was drilled in the shell over the heart of each mussel,

the mantle was left intact, and injection was made directly into the heart. The animals were immersed, and blood was withdrawn from the heart at stated intervals during 30 minutes after the injection. Colorimetric measurements were made according to the procedure described above for the crayfish, except that in four clams in the second series a Coleman spectrophotometer was used for greater accuracy of measurement. Dye was usually injected in 0.1-mg. amounts and sodium thiocyanate in 2.36-mg. amounts in the 1948 series, and in 0.046–0.056-mg. amounts in the 1949 series. When both SCN and T-1824 volumes were measured in the same individuals, the two substances were injected simultaneously in the 1948 series and were injected and determined successively (SCN first) in the 1949 series.

Two difficulties arose in interpreting the results: uncertainty as to the time of complete mixing, and the measurement of true flesh weight or volume. Of the thiocyanate curves (Fig. 2), eight showed continuously declining concentration, while eighteen showed a fairly constant concentration during 2–20 minutes. Of the dye curves, sixteen showed declining concentration, seven showed a maximum some minutes after injection, and four were constant. Probably these differences in the time-concentration curves reflect circulatory differences, but no correlation with heart rate was found. The curves with a maximum concentration several minutes after injection indicate that considerable time is needed for mixing, and on this basis the maximum concentration was taken for fluid-volume estimation. Volumes so calculated were not significantly different from volumes obtained from the flat curves.

In the 1948 series each intact clam was weighed after an experiment, the flesh was removed, and then the shell weight was subtracted from total weight to give

TABLE 1

T-1824 AND NACNS VOLUMES IN CRAYFISH (*Cambarus virilis*)

Body Weight (Gm.)	T-1824 (Ml.)	Volume (Per Cent Body Weight)	NaCNS (Ml.)	Volume (Per Cent Body Weight)	Single or Double Determination
10.5	3.7	35.3	.....	.....	S
15.0	4.34	28.7	.....	.....	S
16.3	6.99	42.8	7.76	47.6	D
16.5	5.23	31.5	.....	.....	S
19.0	2.63	13.8	3.88	20.4	D
21.0	4.34	20.7	.....	.....	S
22.2	.....	.....	3.88	17.5	S
24.0	7.7	32.1	9.48	39.5	D
26.6	2.63	9.9	4.41	16.6	D
26.8	3.89	14.5	.....	.....	S
27.1	.....	.....	5.17	19.1	S
27.4	.....	.....	6.47	23.6	S
29.0	6.84	23.5	.....	.....	S
29.4	.....	.....	6.47	22.0	S
36.5	8.69	23.8	.....	.....	S
37.0	.....	.....	11.1	30.0	S
39.2	.....	.....	9.88	25.2	S
48.2	.....	.....	9.88	20.5	S
Mean	.....	25.1	.....	25.6	.....

the flesh weight. In the 1949 series the following measurements were made: total weight of clam, weight after draining water from mantle cavity by an opening at posterior end, weight of flesh, weight of shell, and volume of flesh by water displacement. It is difficult to drain all the water, particularly that retained by the gills. In twelve clams the water drained from the mantle cavity after an experiment averaged 11 per cent of the total weight; this was approxi-

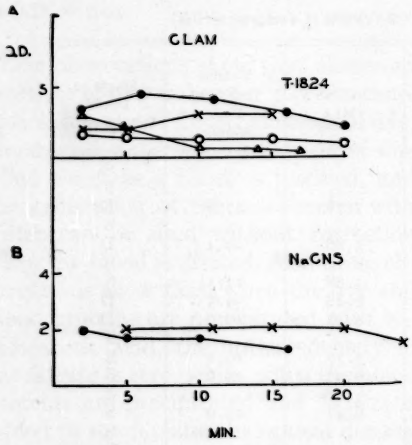


FIG. 2.—Typical time-concentration curves for fresh-water mussels selected to show the different shapes of curve observed. *O.D.*, relative optical density. *A*, T-1824; *B*, NaCNS.

mately one-fourth the amount drained from clams fresh from the stock aquariums. This water weight was subtracted from the body weights used in the 1948 series, and the relative blood volumes increased proportionately. In fifteen clams the specific gravity averaged 1.08 (limits 1.03–1.13), while the specific gravity of the blood is 1.0026 (Ellis, Meirick, and Ellis, 1930). Blood volume as percentage of body volume is, therefore, 7.7 per cent greater than as percentage of body weight.

Detailed data for some typical animals of the 1949 series and summaries of both series are given in Tables 2 and 3. The body weights and volumes for the 1948 series were calculated as stated above and were measured for the 1949 series. The high variance of the data is indicated by standard deviations. The mean SCN volumes were lower in the second, than in the first, series, while the reverse was true for mean T-1824 volumes. However, the standard deviations are large within each series, thus indicating much individual variability. The average thiocyanate volumes and dye volumes are essentially the same, indicating that each substance measures a similar fluid compartment. This average fluid volume of slightly less than 10 per cent of body volume seems unexpectedly low for an animal with open circulation.

It was found that the dye T-1824 complexes with protein, as in crayfish blood; but, since the blood protein concentration is low (0.074 per cent) (Lustig and Ernst, 1937), it seemed possible that there might be some loss of dye, although such loss would increase the apparent blood volume. As a check, the dye was complexed with mammalian plasma protein before injection. Human plasma was used in eight of eighteen determinations in the 1948 series and horse plasma in eight of thirteen in the 1949 series. There was overlap of T-1824 volumes with and without added proteins, and therefore all measurements are included in the figures of Tables 2 and 3. Previous complexing with protein alters neither the time-concentration curve nor the dye dilution volume.

Fresh-water mussels are sluggish animals, and blood passes from arteries into numerous hemocoelic sinuses. It is possible that there is much variation in circulatory activity, that blood flow through some of the sinuses can be interrupted and the blood shunted through

restricted channels. If so, the true hemolymph volume would be larger than the measured values, since in sluggish individuals the dye and thiocyanate would not be completely mixed throughout the hemocoel. Animals were classed as "active" if the heart beat regularly and as "sluggish" if the beat was slow or occasional. In the 1949 series, activity was more nearly uniform, but in the first series in five active animals the T-1824 volume averaged 7.9 per cent of body weight, while in six sluggish animals it was 4.9 per cent of the body weight.

In an attempt to check the dilution volumes, blood was drained from a cut in the heart while gentle pressure was applied to the viscera. It is difficult to drain blood only, and undoubtedly there was variable contamination with water retained between the lamellae of the gills. In five of eight mussels the drained "blood" averaged 96 per cent of the volume measured by T-1824 dilution. In the other three the drained fluid volume was much greater, and blood was certainly diluted by water. It is unlikely that all the blood in the body could be drained from the heart; yet the quantities obtained support the conclusion reached by the dilution method that the blood volume may be approximately 10 per cent of the body volume.

Frogs.—Ten grass frogs were used (Table 4), four with T-1824 alone, two with NaCNS alone, and three with both substances simultaneously. In two bullfrogs dye and thiocyanate spaces were

TABLE 2

THIOCYANATE VOLUMES AND T-1824 VOLUMES IN FRESH-WATER MUSSELS

(Typical Data from 1949 Series)

Species	SCN Volume			Dye Volume			Weight	Volume	Shell Weight (Gm.)
	Ml.	% Wt.	% Vol.	Ml.	% Wt.	% Vol.	Gm.	Ml.	
Amblema costata	2.4	4.6	5.1	7.9	15.0	16.4	52.5	48	184
A. costata	4.2	5.1	5.6	9.2	11.3	12.3	81.9	75	267
A. costata	3.1	6.1	6.6	5.5	10.9	11.7	50.7	47	100
Anadonta grandis	1.1	2.9	2.8	6.7	17.3	16.9	39.2	.....	114
Lampsilis fasciola	4.3	9.6	11.0	4.4	7.7	11.3	45.0	39	34
Anadonta grandis	5.4	14.0	16.3	3.8	9.9	11.5	38.5	34	34
Not determined	4.3	4.8	5.1	16.4	16.8	19.3	90.2	85	232
Not determined	1.4	4.5	4.8	5.5	17.2	18.3	32.0	30	63

TABLE 3

AVERAGE THIOCYANATE AND DYE VOLUMES IN FRESH-WATER MUSSELS

Series	Average SCN Volume					Average T-1824 Volume				
	No. of Animals	% Wt.	σ	% Vol.	σ	No. of Animals	% Wt.	σ	% Vol.	σ
1948	15	9.25	4.8	9.96	.....	17	6.10	2.5	6.58	.....
1949	10	6.2	3.4	7.3	3.8	13	10.6	5.2	14.0	5.2
Total average	.....	8.1	.....	9.0	.....	.....	8.0	.....	9.5	.....

measured simultaneously. The grass frogs ranged in weight from 13.5 to 26 gm., and the bullfrogs weighed 435.5 and 504 gm. The frogs were kept moist and were restrained, ventral side uppermost, during an experiment.

The grass frogs were injected with 0.2 ml. of T-1824 (0.1 mg.) or with 0.1 ml. of NaCNS (2.36 mg.). Injection was made intracardially. From the small frogs 0.05- or 0.1-ml. samples were withdrawn from the heart at stated intervals during 30 minutes after injection. Time-concentration curves were made, and volumes were computed by extrapolation to zero time (Fig. 3).

It was found that the citrate used to prevent clotting formed a colored complex with $Fe(NO_3)_3$, for which a correction had to be made. The plasma was diluted one hundred times, and a comparable amount of frog plasma was added to the blank against which colorimetric readings were compared.

Results are given in Table 4. Hematocrits averaged 13.9 per cent, and the T-1824 volumes averaged 7 per cent of body weight. Blood volumes varied from 6.4 to 10.6 per cent, with an average of 8.2 per cent of body weight, while the thiocyanate volumes were 24–30 per cent, with a mean of 27.3 per cent of body weight. Values for the two bullfrogs were similar except for an unusually large thiocyanate space in one. The blood volume is similar to the value of 8.7 per cent obtained by vital red dilution by Conklin (1930) in frogs. The ratio between blood and thiocyanate volume is, therefore, similar in frogs and mammals.

TABLE 4

BLOOD VOLUME CALCULATED FROM T-1824 VOLUME AND HEMATOCRIT AND NACNS VOLUME OF FROGS

Body Weight (Gm.)	Hematocrit (% Cells)	Blood Volume		T-1824 Volume (% Body Weight)	NaCNS Volume (% Body Weight)
		Ml.	% Body Weight		
	Grass Frogs				
13.5	16.6	1.04	7.7	6.4	.....
15.5	16.0	1.49	9.6	8.1	.....
16.0	12.5	1.70	10.6	9.3	28.2
17.0	14.3	1.28	7.5	6.5	.....
19.0	12.5	1.31	6.9	6.0	.....
20.2	13.0	1.71	8.5	7.4	27.5
21.5	12.5	.....	.....	.....	27.6
22.5	13.6	.....	.....	.....	.....
23.5	12.5	1.51	6.4	5.6	23.6
26.0	15.2	.....	.....	.....	29.8
Average	13.9	.....	8.2	7.0	27.3
	Bullfrogs				
436	16.0	37.2	8.5	7.2	29.8
504	15.0	52.4	10.4	8.8	47.9

Yellow bullheads (*A. natalis*).—Meas-

urements of T-1824 volume only were made on two bullheads, of NaCNS volume only in two, and of both simultaneously in four. Each fish was placed in a weighed amount of water in a jar and weighed; weights varied from 51.5 to 328 gm. The fish were anesthetized in ice water, then were tied, ventral side up, in a metal trough, and the trough was immersed so that the gills were covered

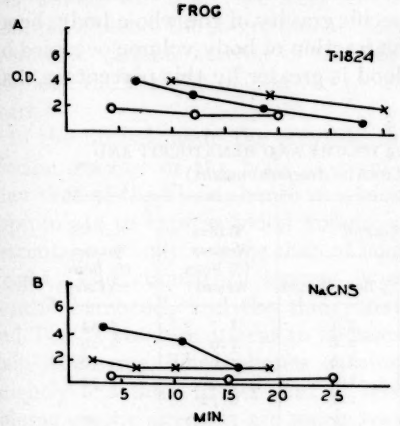


FIG. 3.—Selected typical time-concentration curves for frogs. *O.D.*, relative optical density. Time in minutes after injection. *A*, T-1824; *B*, NaCNS. Open-circle curves are for bullfrogs, others for grass frogs.

with water. Injection was into the heart, which was reached by puncturing the skin in the midline just caudal to the operculum. Only 0.2 ml. (0.1 mg.) of T-1824 and 0.1 ml. (2.36 mg.) of NaCNS were injected. Blood samples of 0.1 ml. were removed from the heart at stated intervals. Blood was citrated, plasma separated, and the concentrations of dye and NaCNS measured colorimetrically in the same manner as with frog blood. Typical time-concentration curves are given in Figure 4. These were extrapolated to the injection time, and the volumes were calculated. Diluted plasma was used in the colorimeter blanks.

Results are given in Table 5. The average plasma (T-1824) volume was only 1.25 per cent and blood volume 1.8 per cent of body weight. The average thiocyanate volume was 4 per cent of body weight. The values given in Table 4 are uncorrected for the injected fluid; since the volumes are small, such correction would reduce the blood and thiocyanate volumes by one-twentieth to one-tenth. These T-1824 and NaCNS volumes in bullheads are much smaller than in mammals or frogs but are comparable with earlier data on bony fish (Reichert and Brown, 1909; Derrickson and Amberson, 1934). Inspection of Table 5 shows an inverse correlation of fluid volume with body size, the larger fish having smaller relative dye and thiocyanate volumes

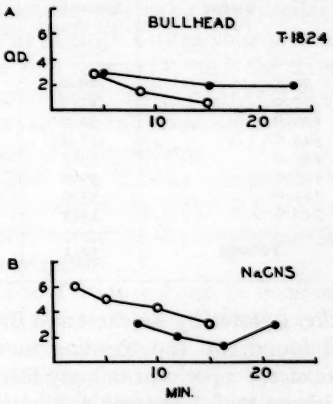


FIG. 4.—Typical time-concentration curves for bullheads. *O.D.*, relative optical density. *A*, T-1824: *B*, NaCNS.

than the smaller fish. A high rate of circulatory turnover is indicated.

DISCUSSION

In mammals the dye T-1824 combines with plasma albumin and the observed dilution indicates a plasma volume of 4–5 per cent and a blood volume of 8–10 per cent. Thiocyanate gives an approximate measure of total extra-cellular fluid, vol-

umes varying from 25 to 35 per cent. Thus the ratio of thiocyanate volume to blood volume in mammals is 3 or 4 to 1. In the frog the ratio of thiocyanate space (extra-cellular fluid) to blood is 3.3 to 1. The blood volume obtained by T-1824 dilution (8.2 per cent of body weight) is of the same order as by other methods, 6.42 per cent by bleeding method (Derrickson and Amberson, 1934) and 8.74 by dilution (Conklin, 1930). In the bullhead, however, blood volume is much less, an average of 1.8 per cent. Low blood volumes are characteristic of bony fish. teins and is therefore removed from the blood when the proteins are precipitated. The crayfish has an open circulatory system—the hemocoel; hence the dye and thiocyanate volumes should be identical. An average value of 25.1 per cent for T-1824 volume and 25.6 per cent for thiocyanate space on the basis of body weight proves such a prediction to be true. In *Cambarus* the specific gravity of the blood is about 20per cent less than the specific gravity of the whole body; hence the fraction of body volume occupied by blood is greater by this percentage than

TABLE 5

BLOOD VOLUME CALCULATED FROM T-1824 VOLUME AND HEMATOCRIT AND THIOCYANATE VOLUME OF BULLHEAD (*Ameiurus natalis*)

Weight (Gm.)	Hematocrit (% Cells)	Blood Volume		T-1824 Volume (% Body Weight)	NaCNS Volume (% Body Weight)
		Ml.	% Body Weight		
51.5	20.0	1.09	2.1	1.7	5.34
77.0	25.0	1.99	2.6	1.9	6.28
109.0	33.0	2.63	2.4	1.6	3.56
143.0	33.3	2.07	1.4	0.96	2.71
145.0	33.3	1.58	1.1	0.72	.....
193.0	30.0	.....	.....	.....	2.90
325.0	33.0	.....	.....	.....	3.2
328.0	33.3	3.6	0.96	0.64	.....
Average	30.1	.....	1.77	1.25	4.0

Welcker (quoted by Reichert and Brown, 1909) found, by the bleeding method, values of 1.5–2 per cent in bony fish, and Derrickson and Amberson (1934) found in *Tautoga* a blood volume of 1.5 per cent body weight. In the bullhead the ratio of thiocyanate volume to blood volume is 2.2, slightly less than in mammals and frogs. Blood volumes in elasmobranch fish more closely resemble volumes in frogs (Derrickson and Amberson, 1934; Martin, 1947).

In the crayfish the protein fractions of the blood have not been completely identified; however, it is of interest to note that T-1824 complexes with blood pro- the fraction of body weight which is blood weight, giving 30.7 per cent of the body volume as blood. The NaCNS volume in *Cambarus* is of the same order of magnitude as that reported for *Carcinus* (37 per cent, Nagel, 1934) and *Eriocheir* (33 per cent, Krogh, 1939).

Sodium thiocyanate in the amount necessary for measurement (2.36 mg.) and in minimal volume (0.1 ml.) proved toxic to the crayfish. The heart often contracted with a violent spasm, remained contracted, and showed a few weak beats 10–15 minutes following injection. Therefore, considerable time elapsed before the NaCNS reached max-

imal concentration in the abdominal sinus, where the sampling was done. That the crayfish heart is sensitive to NaCNS probably results from its neurogenic nature. Thiocycnate is an ion which has been demonstrated to be very toxic to the nervous system of the crayfish (Prosser, 1943). The wide variance of dye and thiocyanate space of 9.9–47.6 per cent is probably due to a number of factors, among which are the sluggish circulation of blood, the stagnation of blood in sinuses, variable activity of the crayfish, and the toxicity of NaCNS to the heart.

In the mussel, as in the crayfish, the specific gravity of the body is greater than that of the blood; hence it is more appropriate to express blood volume as percentage of body volume than of body weight. The circulatory system is an "open" hemocoel, and the thiocyanate and T-1824 volumes appear to be essentially the same. The volumes obtained (slightly less than 10 per cent of body volume on the average) are much lower than anticipated. Despite high variability, almost no values were so high as the relative volumes in the crayfish, and volumes obtained by bleeding and by dye dilution were of the same order. However, active animals tend to show larger dye volumes than do sluggish ones, and shunting of some of the sinuses is possible. The extreme individual variability and the variety of time-concentration curves indicate differences in circulatory activity. Also it is difficult to drain all the water from mantle and gill cavities, an error which may make measurements of true body weight low and blood volume high. It would be of interest to make similar blood-volume measurements on other mollusks which are more active and and which do not retain so much water. Possibly mollusks do not have so much circulating fluid as crustaceans do. On the basis of blood-volume measurements, closed circulatory systems have a higher utilization of blood than open ones do, the circulation in bony fish showing more turnover than that of other vertebrates.

SUMMARY

1. The blood volume of crayfish, fresh-water mussels, frogs, and bullheads was measured by dilution of the dye T-1824, and "extra-cellular space" was estimated by dilution of NaCNS.

2. In crayfish the specific gravity of the body is approximately 20 per cent above that of blood and in mussels 6 per cent above; hence blood volumes as percentage of body volume are larger than as percentage of body weight, whereas in mammals the two values are similar.

3. The average T-1824 volume in crayfish is 25.1 per cent, and the average thiocyanate volume 25.6 per cent of body weight, or a total blood volume of 30.5 per cent of body volume.

4. The dye T-1824 complexes with blood protein in the crayfish and mussel. NaCNS is toxic to the neurogenic heart of the crayfish.

5. The T-1824 volume of fresh-water mussels averaged 9.5 and the thiocyanate volume 9.0 per cent of body volume, or about 8 per cent of body weight. The measured fluid volumes of active mussels were greater than those of sluggish individuals, the individual variability was very large, the estimate of body weight free from mantle water was difficult, and it is possible that the true blood volume is somewhat greater than the measured values. However, the volume of drained blood was similar, and the dye and thiocyanate appear to measure the same fluid volume; hence it is unlikely that the circulating fluid is greatly above 10 per cent.

6. In grass frogs an average T-1824 volume of 7.0, NaCNS volume of 27.3, and blood volume of 8.0 per cent of body weight were obtained.

7. In bullheads the average T-1824 volume was 1.25, blood volume 1.77, and thiocyanate volume 4.0 per cent of body weight.

8. It is concluded that thiocyanate and T-1824 measure similar volumes in animals with open circulations and that blood volume is less in closed than in open circulatory systems.

LITERATURE CITED

CONKLIN, R. E. 1930. The formation and circulation of lymph in the frog. II. Blood volume and pressure. Amer. Jour. Physiol., 95:91–97.

DERRICKSON, M. B., and AMBERSON, W. R. 1934. Determination of blood volume in the lower vertebrates by the direct method. Biol. Bull 67:329.

ELLIS, M. M.; MEIRICK, A. D.; and ELLIS, M. D. 1930. The blood of North American fresh-water mussels under normal and adverse conditions. Bull. Bur. Fish., 46:509–46.

GREGERSON, M. E., and STEWART, J. 1939. Simultaneous determination of the plasma volume with T-1824, and the "available fluid" volume with sodium thiocyanate. Amer. Jour. Physiol., 125: 142–52.

KRIEGER, H.; STORAASLI, J. P.; FRIEDELL, H. L.; and HOLDEN, W. D. 1948. A comparative study blood volume in dogs. Proc. Soc. Exper. Biol. and Med., 68:511–15.

KROGH, A. 1939. Osmotic regulation in aquatic animals. Cambridge: Cambridge University Press.

LUSTIG, B.; ERNST, T.; and REUSS, E. 1937. Die Zusammensetzung des Blutes von Helix pomatia bei Sommer und Wintertieren. Biochem. Zeitschr., 290:95–98.

MARTIN, A. W. 1947. The blood volume of some elasmobranchs. Fed. Proc., 6:164.

NAGEL, H. 1934. Die Aufgaben der Exkretionsogane und der Kiemen bei der Osmoregulation von Carcinus maenas. Zeitschr. f. vergl. Physiol., 21:468–9 .

PROSSER, C. L. 1943. An analysis of the action of salts upon abdominal ganglia of crayfish. Jour. Cell. and Comp. Physiol., 22:131–45.

REICHERT, E. T., and BROWN, A. P. 1909. The differentiation and specificity of corresponding proteins and other vital substances in relation to biological classification and organic evolution. Pub. Carnegie Inst. Washington, 116:1–338.

SOME EFFECTS OF INORGANIC SALTS ON THE BLOOD SPECIFIC GRAVITY AND TISSUE FLUIDS OF THE BLUEGILL LEPOMIS MACROCHIRUS RAF.[1]

ROLAND ABEGG[2]

Northwestern State College, Natchitoches, Louisiana

IN ORDER to control adequately and fairly the purity of streams and other public bodies of water subjected to industrial pollution, it is necessary to know what substances are toxic to their contained fauna, the concentrations at which they are effective, and the mode of their action. The present paper considers the effects of some inorganic salts on the internal water balance of the bluegill, *Lepomis macrochirus* Raf.

[1] This paper represents portions of a dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the Louisiana State University, under the direction of Dr. George H. Mickey, Department of Zoölogy.

[2] This work was done under a graduate research fellowship sponsored by the Louisiana Refiners' Waste Control Council. The writer is also indebted to the following agencies and people: Louis Cusachs, Frank J. Coogan, and James Brown, of the Louisiana Department of Wildlife and Fisheries; A. J. Wilson, superintendent of the United States Fish Culture Station, Natchitoches, Louisiana; James E. Williams, Jr., Junior Research Fellow; Dr. G. H. Mickey, formerly at Louisiana State State University, for his guidance and constructive criticisms during the progress of this investigation.

Scott (1910) showed that the blood specific gravities of marine teleosts varied directly with the concentration of the sea salts in the environment. Westfall (1943) showed that in the goldfish, *Carassius auratus* (L.), the specific gravity of the blood dropped during rapidly developed anoxia but that this drop could be prevented by placing the fish in a 1 per cent sodium chloride solution. The effects of varying the concentrations of sea salts and of sodium chloride on the weight of fishes is recorded in numerous papers. In each case the gain or loss reported was for the whole fish. As early as 1871, Bert thought that the major tissue involved was the musculature, and Smith (1932) postulated that the tissue-water-protein relationship is concerned in the fundamental steady state of the vertebrate. It is not certain how much of the water in fish muscle is "free water," but, according to Hill (1930), the tissue water of mammalian muscle is all "free water" as defined by Gortner (1938). For the present study it will be assumed that this same condition obtains in the fish.

In consideration of the foregoing, it was decided that the changes in the specific gravities of the blood and in the hydration of the muscle tissues of fish subjected to various salt solutions would be a productive line of investigation.

METHODS AND MATERIALS

The methods employed in setting up the testing conditions were, in general, those proposed by Hart, Doudoroff, and Greenbank (1945). Exception was taken to the recommended size of the test animal, 4–10 cm. Sufficient quantities of blood could not be obtained from fish of this small size. Therefore, fish weighing 20–35 gm. were utilized. However, in any one test the range of weights of fish used was less than 10 gm.

THE TEST ANIMAL

The bluegills were obtained from the United States Fish Culture Station, located on the Cane River Lake at Natchitoches, Louisiana, and were transported to Baton Rouge in trucks especially designed for the transportation of fish. Transferring first directly from the Cane River transportation water, pH 7.3, to the outdoor holding-tank water (Baton Rouge tap water), pH 8.6, resulted in the mortality of about 90 per cent of the fish of the first shipments. The pH of the holding-tank water was therefore adjusted with HCl to within 0.4 units of the transportation water. The temperature was also adjusted to $\pm 3°$ C. of the transportation water. Tap water was then slowly sprayed into the tanks, so that, after 24–48 hours the tank water had assumed the temperature and pH of the tap water. Following the adoption of this procedure, no more fish died in these tanks.

The fish were fed daily a commercial, pellet-form, goldfish food, in quantities that they could consume immediately. This greatly reduced the fouling of the water, especially in the laboratory acclimatizing tanks.

ACCLIMATIZING THE TEST ANIMAL

Several days before a test was to be made, a group of fish was transferred to the laboratory tanks, in which the temperature had been adjusted to that of the holding tanks. The temperature of the water was then allowed to come to equilibrium ($21° \pm 1°$ C.) with the temperature of the air-conditioned laboratory ($22° \pm 1°$ C.). Equilibrium was reached in 5–8 hours.

After the fish had been acclimated to temperature for 24–48 hours, from 26 to 30 fish were roughly graded for size and transferred to reference water (see below)

of the same temperature, for a minimum acclimatization period of 4 days.

PREPARATION OF REFERENCE WATER

The term "reference water" designates a water containing all the major ions in concentrations and proportions typical of a mean surface water of the United States[3] (Hart, Doudoroff, and Greenbank, 1945). In addition, the solution contains minor mineral elements in concentrations typical of natural waters. Reference water was prepared in the laboratory, using distilled water and analytical grade chemicals.

To satisfy the large demand for reference water, it was made up in 100-gallon lots. Distilled water was obtained from a 5-gallon-per-hour steam-heated still, which produced a distillate of very high quality, 350,000–400,000 ohm specific resistance at 25° C.

The maximum and minimum limits of chemical composition to which this water must conform are set forth by Hart, Doudoroff, and Greenbank (1945) as shown in the accompanying tabulation.

	P.p.m.
Total alkalinity (as $CaCO_3$)	60–120
Total hardness (as $CaCO_3$)	75–150
Sulphates (as SO_4)	20– 50
Dissolved solids (maximum)	<500

Reference water, as used in this laboratory, was not entirely satisfactory. Fish placed in this water showed definite signs of distress, expressed as an orientation defect, the fish darting nervously about the tank, with their bodies inclined at a 45° angle, head down. After a few hours the fish became adjusted to the new water. After 3 or 4 days the fish again showed signs of distress, congregating in a corner of the tank, behaving sluggishly, and with their bodies at a 45° angle, but with their heads at the the surface. This problem requires further attention if reference water is ever to be accepted as a standard laboratory reagent.

TEST CONDITIONS

Four 5-gallon round glass battery jars were used in each test: two for the test solutions and two for the reference water of the control group. Each jar contained 12 liters of solution or water. The four containers were accommodated in the constant-temperature bath simultaneously, and the solutions and water were aerated until they had reached the test temperature, 22°.2 ± 0°.2 C. To minimize evaporation and consequent alteration of the strength of the solutions, each jar was covered with a glass plate. Aeration of the solutions was accomplished by passing the air through carborundum air dispersers after oil, water, and grit had been removed by a porous stone filter.

The strengths of the test solutions used were those which had been found to yield the 24-hour median tolerance-limit concentrations.[4] These values were determined in this laboratory by James E. Williams, Jr. (1948), and in part by the author (1947*a*). The test conditions and procedures for making these determinations were identical with those outlined in the present paper except that this series of tests involved at least three determinations at different concentrations for each of the chemicals and the fish were 4–10 cm. in length. When three concen-

[3] The values for the various ions which make up a mean United States surface water "are estimates based on the data of Clark (1924), and they show the approximate median concentrations of each of the more important inorganic components of surface waters . . . of the United States" (Hart, Doudoroff, and Greenbank, 1945). The author is indebted to the Waste Control laboratory of the Atlantic Refining Company, Philadelphia, for the formula used in making up this water.

[4] The 24-hour median tolerance limit (24-hour Tlm) is the concentration of a chemical or solution which half the test animals can survive for a period of 24 hours. It may be expressed as either percentage or parts per million.

trations had been tested in which the mortality in 24 hours was not more than 80 per cent and not less than 20 per cent, the data were plotted on semilogarithmic co-ordinate paper. The percentage of mortality was plotted on the arithmetic scale and the concentration on the logarithmic scale. The 24-hour median tolerance-limit concentration was then estimated by straight-line interpolation.

These 24-hour median tolerance-limit concentrations were not in every case so lethal to the larger fish used in the present study as they were to the smaller fish used in the median tolerance-limit tests described above. However they were tested, toxic concentrations and, theoretically, the tests were made using the same base.

The solution characteristics are summarized in Table 1. The concentrations for each chemical listed are given in parts per million and as mols per liter. The osmotic-pressure figures expressed in liter atmospheres represent the pressures exerted by the concentrations of the chemicals in question plus the pressure of the reference water used as the diluent, i.e., the total calculated osmotic pressure to which the fish were subjected.

To reduce the fouling of the solutions in the test containers, the fish were not fed during the test or during the 24-hour period preceding the beginning of the test. For the same reason the fish were rinsed in clean reference water before being placed in the test solutions. Acclimatized fish (see p. 125) were selected at random from the rinsing bath and placed alternately in the test and in the control containers until there were 6 fish in each of the four containers.

TABLE 1

TEST SOLUTION CHARACTERISTICS

Chemical	P.p.m. of Chemical Used	Mols per Liter of Chemical Used	Calculated Osmotic Pressure of Test Solution in Liter Atm.*	Specific Resistance in Ohms at 22°.5 C.†	pH	Temperature ° C.
Na_2SO_4	17,500	0.1247	3.04	55	7.9	22.5
NaCl	14,100	0.2428	5.90	45	8.5	22.5
$CaCl_2$	8,400	0.0772	1.89	70	8.3	22.5
Na_2HPO_4	5,800	0.0425	1.04	170	8.0	22.5
KCl	5,500	0.0750	1.81	105	8.7	22.5
Na_2CrO_4	930	0.0073	0.19	520	8.1	22.5
$Na_2Cr_2O_7$	728	0.0044	0.12	105	5.9	22.5
Na_2CO_3	387	0.0053	0.14	1,175	9.6	22.5

* Osmotic pressure due to the ions in the reference water, calculated to be 0.05 liter atm. plus the osmotic pressure due to specific chemical in question.

† The reference water specific resistance at 22°.5 C. was 3,300 ohms.

BLOOD SPECIFIC-GRAVITY DETERMINATIONS

The use of the falling-drop densiometer (Barbour and Hamilton, 1926–27) for measuring changes in the specific gravity of small quantities of fish blood was original with the writer, although later it was discovered that this method had been employed by Westfall (1943). This falling-drop method combines the merits of small sample size, 0.01 cu. mm., with relatively great speed, and, according to Barbour and Hamilton, this method is accurate to 1×10^{-4}.

To obtain the blood, fish were removed one at a time from the test containers, were pithed and weighed, and the heart was exposed with a curved cutting

probe. After the pithed fish had been weighed, the opercula were cut away (see Figs. 1 and 2). An incision was made on each side of the isthmus, penetrating to the parietal pericardium and extending to the junction of the isthmus with the ceratohyal. The narrow connection between the isthmus and the ceratohyal was cut, and the isthmus was raised so that the connective tissue between it and the parietal pericardium could be separated. The isthmus was then cut away

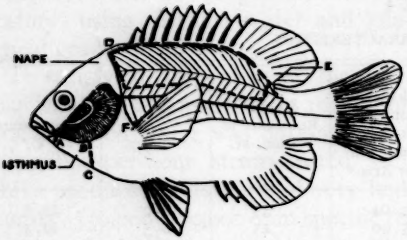


FIG. 1.—Lateral view of fish, showing incisions for the exposure of the heart and the muscle tissue (*Lepomis macrochirus* Raf.).

with a transverse cut which extended from the pericardial chamber posteroventrally, and the pericardial membranes were teased to expose the bulbus. The pericardial fluid was removed with absorbent paper. A $\frac{3}{4}$-inch needle tube (broken from a 23-gauge hypodermic syringe needle) held in a bulldog clamp was used to pierce the bulbus arteriosus. The action of the heart pumped the blood out through the needle tube, which was held so that the blood could be collected on a paraffin block. When sufficient blood had been collected, the fish was covered to prevent excessive surface evaporation while the blood specific-gravity determination was being made.

TISSUE-FLUID DETERMINATIONS

After the completion of each specific-gravity determination, a strip of dorsal muscle tissue was removed from one side of the fish. This was accomplished as follows: After the removal of the scales, two incisions were made (see Fig. 1, *DE* and *DF*). The skin was grasped at *D* and stripped back, exposing the dorsolateral musculature. A strip of this tissue was removed with a scalpel, care being taken to exclude neural spines, ribs, or other tissues. The tissue was weighed in a tared, covered weighing dish on an analytical balance and placed in an electric oven, thermostatically controlled at a temperature of 110° C. After 24 hours the tissue was placed in a desiccator to cool and was then reweighed. The results were calculated and expressed as percentage of water of the wet-tissue weight.

EXPERIMENTAL RESULTS

In any experiment the ideal situation is that in which the tissue to be tested is from the same animal, before and after

FIG. 2.—Ventral view of fish, showing heart exposed in preparation for obtaining blood for a density determination (*Lepomis macrochirus* Raf.).

being subjected to the experimental conditions. In the determination of the changes in the percentage of tissue fluids, this ideal was patently impossible. At the outset of the study, however, it was hoped that a technique could be developed by which 2 drops of blood could be obtained from the same fish, 24 hours apart: before and after the fish had been exposed to the test conditions. This was done by hypodermic puncture of the sinus venosus just anterior to the pec-

toral girdle. Although all fish subjected to this treatment survived, the specific-gravity values of the second drop of blood were considerably lower, having dropped as much as 8.8×10^{-3}, thus precluding the use of this technique.

The data for all the salt solutions employed in this study are summarized in Table 1. The pertinent figures for the experimental results are summarized in Table 2.

water balance of the fish exposed to these chemicals for 24 hours. The tissue-fluid and blood specific-gravity mean values of the test groups were not significantly different from the mean values of the control groups. This is indicated in Table 2 by the notation "N.S." in the columns for "*P* Value" and "Per Cent Change." In each case all the fish survived the 24-hour test period, and it is possible that the increased tolerance of the fish to

TABLE 2

STATISTICAL DATA ON TISSUE-FLUID AND BLOOD SPECIFIC-GRAVITY VALUES FOR TEST AND CONTROL GROUPS OF FISH FOR EACH CHEMICAL TESTED

Chemical	Tissue Fluids						Blood Specific Gravity				
	No. of Fish	Hours Exposed	Mean Per Cent Tissue Fluid	Standard Deviation	P Value*	Per Cent Change†	No. of Fish	Mean Density	Standard Deviation	P Value*	Per Cent Change†
Na_2SO_4....	10 C‡	24	81.27	1.4213	.....	.....	10 C	1.0441	0.006660	.....	.....
	10 T§	24	79.88	1.7567	N.S.	N.S.	10 T	1.0453	0.005663	N.S.	N.S.
NaCl.....	11 C	7	80.92	0.8152	.....	.....	11 C	1.0417	0.003395	.....	.....
	7 T	7	78.13	0.4822	0.01	−3.45	7 T	1.0363	0.003187	0.01	−0.518
$CaCl_2$.....	10 C	24	81.45	0.9463	.....	.....	10 C	1.0386	0.002966	.....	.....
	11 T	24	80.36	0.8836	0.02	−1.34	10 T	1.0392	0.003567	N.S.	N.S.
Na_2HPO_4...	10 C	24	81.64	1.0004	.....	.....	10 C	1.0428	0.003252	.....	.....
	10 T	24	82.42	1.2204	N.S.	N.S.	10 T	1.0427	0.003129	N.S.	N.S.
KCl.....	10 C	24	80.73	1.7355	.....	.....	9 C	1.0389	0.003597	.....	.....
	8 T	24	78.86	0.5097	0.05	−1.87	8 T	1.0377	0.004291	N.S.	N.S.
Na_2CrO_4...	10 C	24	81.22	0.8267	.....	.....	10 C	1.0441	0.001451	.....	.....
	10 T	24	81.63	0.6610	N.S.	N.S.	10 T	1.0452	0.006406	N.S.	N.S.
$Na_2Cr_2O_7$...	10 C	13	80.30	0.7457	.....	.....	10 C	1.0399	0.011361	.....	.....
	11 T	13	82.24	1.4419	0.01	+2.42	9 T	1.0372	0.003501	0.05	−0.375
Na_2CO_3....	10 C	7	81.47	0.8192	.....	.....	10 C	1.0410	0.003419	.....	.....
	10 T	7	82.48	0.8361	0.02	+1.24	10 T	1.0412	0.003170	N.S.	N.S.

* Probability based on Fisher's *t*-test values. "N.S." denotes no significant change.
† Calculated on the basis of the control values equaling 100 per cent.
‡ Control fish. § Test fish.

The fish were exposed to the solutions for 24 hours except in those cases in which it became evident that the test animals would not survive, when the testing was begun immediately (see Table 2). In this respect the results are not strictly comparable.

The solutions of sodium sulphate, sodium chromate, and sodium orthophosphate, dibasic, had no effect on the

these chemicals was related to the larger size of test fish used in this study.

Potassium chloride and calcium chloride solutions caused the test fish to lose tissue fluids, although the blood specific gravities were not significantly different from their respective control groups. The test group of fish in the potassium chloride solution showed a loss of 1.87 per cent tissue fluid, and the fish in the cal-

cium chloride solution lost 1.34 per cent tissue fluids.

The test group of fish exposed to the sodium carbonate solution for 7 hours showed a significant gain of 1.24 per cent tissue fluids over the control group. No significant change in blood specific-gravity values was demonstrated.

The tissue-fluid and blood specific-gravity mean values of fish subjected to

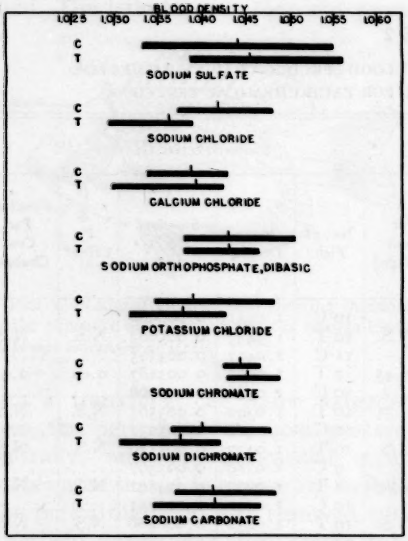


FIG. 3.—Blood density ranges and means of the control and test groups of fish subjected to solutions indicated (*Lepomis macrochirus* (Raf.).

sodium dichromate indicated a significant general hydration of the whole animal. There was a 2.42 per cent increase in the mean percentage of tissue-fluid values of the test group, and the mean blood specific gravity was 0.375 per cent lower than that of the control group.

The test-fish group exposed to sodium chloride exhibited a 3.45 per cent loss of tissue fluids and a 0.518 per cent drop in the mean blood specific-gravity value.

All three of the chloride solutions had a dehydrating effect on the musculature, whereas the solutions of sodium dichromate and sodium carbonate caused the hydration of these tissues.

DISCUSSION

In this laboratory the determination of blood specific gravity was first attempted with large-mouthed bass, *Micropterus salmoides* (Lacépedè), weighing between 5 and 10 gm. (Abegg, 1947*b*). The data from this study indicated that blood densities would reflect the physiological condition of the fish, but it also showed that this method would require the use of groups of fish and that the data obtained would have to be treated statistically. The fundamental premise of the testing procedure also pointed to a statistical treatment of the data. The underlying principle of the 24-hour median tolerance-limit concentration is that fishes exhibit a range of tolerance to changes in the environment. Therefore, in any test using randomly selected test animals, there would be a graded series of responses to the test solutions. Furthermore, as only 50 per cent of the fish die at the 24-hour median tolerance-limit concentration, it might be expected that about 50 per cent of the tested values of the test animals would be still within the range of the normal fish values. The values for the more sensitive fish would spread out and extend beyond one or the other of the extreme values for the control group. This condition was generally exhibited by all the data from all the tests and will be discussed below (see Figs. 3 and 4 and Table 3).

The use of whole-blood specific-gravity values was adopted only after it was impossible to find an anticoagulant which did not change the specific gravity of the blood. Copely and Whitney (1943) showed that the specific gravity of dog, sheep, and human blood increased with the addition of heparin but that this in-

crease was not in linear proportion. Likewise, Barbour and Hamilton (1926) showed that the oxalation of blood caused considerable changes in the specific gravity of blood. The small quantities of blood obtainable from the size of fish used made it impossible to heparinize each sample quantitatively. This precluded the obvious use of plasm specific-gravity values, which would have shown much more accurately the changes in the water balance of the fish. The protein content of the blood could have been determined then also.

The various physiological reactions of the fish to the several salt solutions are difficult to explain unless one assumes that the ions enter the fish's body. There are several possible pathways by which this entrance could be effected: through the skin, gills, and oral membranes and by ingestion. It has been shown (Abegg, 1949*a*) that the salt solutions used in this study altered the mucous covering of the body. In a separate series of tests, fish were placed in salt solutions of the same kinds, and concentrations are reported in this paper. Phenol red was added to these solutions, so that the concentration of the dye was 1:10,000. The fish were removed after 1 hour and rinsed off under running tap water for 3 minutes. The body surfaces of all the fish were stained with the dye, excepting the control fish, which had been in reference water. The dye washed off these fish immediately and completely. The alteration of the mucous covering might then have permitted the ions to penetrate the skin.

In this connection it is interesting to note that the three chlorides caused the dissolution of the mucous covering of the body, and in each case there was a dehydrating effect of the musculature. Sodium carbonate and sodium dichromate each caused an increase in the tissue-fluid content of the musculature and in both cases caused the precipitation of the mucous covering of the body.

Until recently most investigators of fresh-water fish physiology have believed that fresh-water teleosts do not drink water but obtain their required water by endosmosis through the oral membranes. Allee and Frank (1948) have recently reviewed the literature on this subject and demonstrated that the goldfish, *C. auratus* (L.), does drink water. Experiments in this laboratory (Abegg, 1949*a*) using Smith's (1930) phenol red technique, have shown that the fresh-water stenohaline teleost, *L. macrochirus* Raf., also drinks water under normal conditions as well as in solutions of higher osmotic pressures. The same fish used above to demonstrate the effects of the salt solutions on the mucous covering of the body were autopsied to determine whether they had ingested water during their 1-hour exposure to the dyed solutions. The

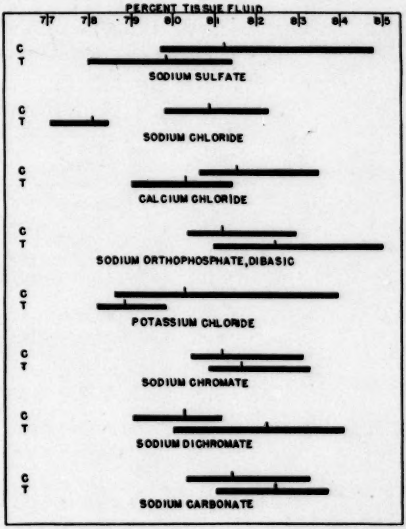


FIG. 4.—Per cent tissue-fluid ranges and means of the control and test groups of fish subjected to solutions indicated (*Lepomis macrochirus* Raf.).

dye was found to be present in all fish, either in the stomach or in the intestinal tract. Dilute sodium hydroxide was used to develop the color of the dye in the stomach. Quantitative measurements were not attempted.

The test fish in the various salt solutions were therefore subjected to both external and internal environmental effects.

TABLE 3

MINIMUM, MEAN, AND MAXIMUM BLOOD DENSITY AND PER CENT TISSUE-FLUID VALUES FOR CONTROL AND TEST FISH (*Lepomis macrochirus* RAF.)

CHEMICAL	FISH	BLOOD DENSITY		PER CENT TISSUE FLUID	
		Range	Mean	Range	Mean
Na_2SO_4.....	C	1.0330–0.0550	1.0441	80.50–84.79	81.27
	T	1.0386–0.0560	1.0453	78.00–83.63	79.88
NaCl.....	C	1.0349–0.0480	1.0417	79.88–82.29	80.92
	T	1.0295–0.0387	1.0363	77.13–78.50	78.13
$CaCl_2$.....	C	1.0336–0.0429	1.0386	80.68–83.46	81.45
	T	1.0299–0.0423	1.0392	79.03–81.45	80.36
Na_2HPO_4.....	C	1.0377–0.0505	1.0428	80.39–82.94	81.64
	T	1.0377–0.0463	1.0427	80.97–85.01	82.42
KCl.....	C	1.0345–0.0450	1.0389	78.63–83.97	80.37
	T	1.0315–0.0424	1.0377	78.22–79.87	78.86
Na_2CrO_4.....	C	1.0423–0.0464	1.0441	80.48–83.10	81.22
	T	1.0428–0.0486	1.0452	80.90–81.63	81.63
$Na_2Cr_2O_7$.....	C	1.0361–0.0475	1.0399	79.09–81.14	80.30
	T	1.0305–0.0412	1.0372	80.07–82.24	82.24
Na_2CO_3.....	C	1.0378–0.0480	1.0410	80.38–83.21	81.47
	T	1.0343–0.0465	1.0412	81.02–83.66	82.48

In Table 3 and Figures 3 and 4 the maximum, minimum, and mean values for the blood specific gravity and percentage of tissue fluid for each test group are compared with these same values for their respective control groups. As indicated in Table 2, not all test-group means were significantly different from their control-group means. However, Figures 3 and 4 showed that in all but one case (sodium orthophosphate, dibasic) the whole test-group range shifted in relation to the control-group range, i.e., the extremes of the test group were the first to be affected by the solutions and in the same direction. It is suggested that these shifts indicated incipient changes which did not proceed to significance. Note again the hydration effect of the three chlorides and compare the shifts of the blood specific-gravity ranges of the test groups with those of the control groups. In each case the extremes have moved toward a lower value. Similarly, compare the shifts in the test-group ranges for the sodium carbonate and sodium dichromate.

While the shifts in ranges presented in the preceding paragraph do not warrant an extensive discussion of the physiological effects of the various salt solutions on fish, certain explanatory postulations are briefly offered for the results obtained.

1. The decreased blood densities as-

sociated with the chlorides were probably due to the influx of tissue fluids in an attempt to keep the chloride level of the blood within the tolerance of the fish.

2. The rise in blood density and the decreased tissue fluids caused by the sodium sulphate solution may have been due to the internal osmotic effects of the solution. Sulphate ions are not readily absorbed from the intestinal tract and hence osmotically drew water from the blood which was in part replaced by tissue water.

3. The general hydration of fish placed in solutions of sodium dichromate and sodium carbonate may be due to the coagulation of the mucous covering of the body, changing the permeability of the skin and gills. The cells themselves might have become completely permeable.

4. The dissolution of the mucous covering of the body caused by the other chemicals (except chromate) used in this study might have affected the permeability of the general body surface and oral membranes as well as the gills, without, however, destroying the epithelial cells of these surfaces, as postulated above in paragraph 3.

SUMMARY AND CONCLUSION

1. Statistically significant changes in the percentage of tissue fluids of the body musculature of the bluegill, *L. macrochirus* Raf., were effected by the solutions of sodium chloride, potassium chloride, sodium dichromate, calcium chloride, and sodium carbonate at the concentrations used in this study and in the length of time the fish were exposed to these solutions.

a) Sodium chloride, potassium chloride, and calcium chloride solutions caused a decrease in the percentage of tissue fluids.

b) Sodium dichromate and sodium carbonate solutions caused an increase in the percentage of tissue fluids.

2. The density of the blood of the test fish was decreased in the solutions of sodium dichromate and sodium chloride at the concentrations used and in the lengths of time the fish were exposed to these solutions. The changes in density effected by these two solutions were significant.

3. The size of fish used in any toxicity-testing program is an important factor in the interpretation of the data. The large test fish (20–35 gm.) used in this study survived the 24-hour test period in solutions of sodium sulphate, sodium chromate, sodium orthophosphate, dibasic, and calcium chloride. The concentrations of these solutions were those which had been shown to yield a 24-hour median tolerance-limit value for small bream (4–10 cm.). The large fish therefore exhibited an increased tolerance to the aforementioned solutions.

4. Sodium sulphate, sodium chromate, and sodium orthophosphate, dibasic, solutions at the concentrations used in this study had no effect on the water balance of the fish exposed to this solution for 24 hours.

5. The bluegill, *L. macrochirus* Raf., drinks water under normal conditions and in the solutions reported in this study.

6. The reference water used in this study was not deemed wholly satisfactory as a dilution water for studies pertaining to the effects of chemical compounds on the physiology of fishes.

7. A mode of action on the fish of each of the salt solutions is postulated.

LITERATURE CITED

ABEGG, R. 1947*a*. Notes on the tolerance of fish to some pure chemicals. Proc. Louisiana Acad. Sci., 11:12.

———. 1947*b*. Blood density of the bass, *Huro salmoides*. Anat. Rec., 99:20.

———. 1949*a*. Some implications from the changes of the mucous covering of fish subjected to salt solutions. Proc. Louisiana Acad. Sci., 12:7–10.

———. 1949*b*. The "normal" bluegill, *Lepomis macrochirus*, under laboratory conditions. Ibid., pp. 10–17.

ALLEE, W. C., and FRANK, PETER. 1948. Ingestion of colloidal material and water by goldfish, *Carassius auratus*. Physiol. Zoöl., 21:381–90.

BARBOUR, H. G., and HAMILTON, W. F. 1926. The falling-drop method for determining specific gravity. Jour. Biol. Chem., 69:625–40.

———. 1927. The falling-drop method for determining specific gravity. Jour. Amer. Med. Assoc., 88:91–94.

BERT, J. P. 1871. Sur les phénomènes et les causes de la mort des animaux d'eau douce que l'on plonge dans l'eau de mer. Compt. rend. Acad. sci., 73:382–85.

COPELY, A. L., and WHITNEY, MARY E. 1943. Density findings with heparin in blood, plasma and serum. Jour. Lab. and Clin. Med., 28:1501–2.

HART, W. B.; DOUDOROFF, P.; and GREENBANK, J., 1945. The evaluation of the toxicity of industrial wastes, chemicals and other substances to fresh-water fishes. Preliminary ed. Philadelphia: Atlantic Refining Co.

HILL, A. V. 1930. The state of water in muscle and blood and the osmotic behavior of muscle. Proc. Roy. Soc. London, 106:477–505.

SCOTT, G. G. 1910. Effects of changes in the density of water upon the blood of fishes. Bull. U.S. Bur. Fish. 1908, 28:1143–50.

SMITH, H. W. 1930. The absorption and excretion of water and salts by marine teleosts. Amer. Jour. Physiol., 93:480–505.

———. 1932. Water regulation and its evolution in the fishes. Quart. Rev. Biol., 7:1–26.

WESTFALL, B. A. 1943. Specific gravity of fish blood during rapidly developed anoxia. Jour. Cell. and Comp. Physiol., 22:177–86.

WILLIAMS, J. E., JR. 1948. The toxicity of some inorganic salts to game fish. Unpublished thesis, Louisiana State University.

INGESTION OF COLLOIDAL THORIUM DIOXIDE BY REPRESENTATIVE MINNOWS FROM THE CHICAGO REGION[1]

PETER FRANK AND W. C. ALLEE
Whitman Laboratory, University of Chicago

THE belief that fresh-water fishes do not swallow water is widespread and has able support (Smith, 1932; Krogh, 1939). Contrariwise, positive indications were reported briefly by Schuster-Woldan (1936) for carp (*Cyprinus carpio*) and tench (*Tinca vulgaris*). His X-ray pictures show that the single specimen tested in each species had engulfed barium sulphate when this quickly settling, X-ray–opaque material was suspended in a sugar solution but not when in water alone. He thought that the sweet taste of the sugar was the stimulus causing swallowing and that fresh-water fish, in general, do not drink. Ellis (1937) found that water containing colloidal dyes and poisons, arsenic among them, was swallowed by fresh-water fishes, usually 48 hours or so after being placed in such a medium.

Allee and Frank (1948) reviewed briefly the controversial history of this subject and present tabular evidence supported by photographs that the goldfish, *Carassius auratus*, ingests colloidal

[1] This investigation was supported in part by a grant from the Rockefeller Foundation. We are also indebted to Dr. Franklin C. McLean for the use of his X-ray equipment; to the Heyden Chemical Corporation, New York, N.Y., for donation of Thorotrast; and to Dr. H. H. Shoemaker, of the University of Illinois, for identification of some of the species of fish.

thorium dioxide (Thorotrast) suspended in a sightly saline solution of dextrin. X-rays clearly revealed this substance in the gut in 29 of the 33 individuals tested; only 2 showed no trace of it. The concentration of the suspension of thorium dioxide was usually 1.3 per cent but ranged from 0.67 to 4 per cent. The duration of time in diluted Thorotrast, usually 24 hours, varied from 1 to 96 hours. The gut was apparently filled with the colloidal suspension (designated "four-plus") in four cases, with exposure before being X-rayed of 5 hours in one instance, and of 24 hours in the other three. In these films there was no suggestion of X-ray opacity in the mouth or on the gills, unlike that found in untreated controls. The available evidence was interpreted as meaning that the small goldfish tested had swallowed water together with its contained colloidal material and that the latter had been concentrated in the gut.

Since the problem of whether freshwater fishes swallow water has prime importance both in water physiology and in food relations, this initial study has been extended in two ways. First, as regards water intake by large goldfish and, second, concerning the generality of the phenomenon among small native freshwater fishes. In the second phase of the investigation we used a series of species representing families of fishes somewhat readily available near Chicago. We limited ourselves to individuals 2–6 inches long, since that size is most suitable for our equipment and allowed efficient use of the supply of Thorotrast. The results obtained both with large goldfish and native fishes are definite and can be reported with accurate brevity. The methods used were essentially similar to those described by Allee and Frank (1948).

The goldfish previously tested ranged between 3 and 5 cm. in body-length, excluding the tail. The life-size roentgenogram reproduced with that of an untreated control in Plate II shows that the single large goldfish used was 127 mm. long from the tip of the upper jaw to the end of the posterior vertebra. It was housed in 1 liter of water in a 1-gallon stoneware jar, 17.5 cm. in diameter. For 4 days it was fed daily, and feces were removed with a syringe pipette. Then enough Thorotrast was gently poured in to make a 1.67 per cent suspension of thorium dioxide; 24 hours later the experimental fish and an appropriate control were anesthetized with chloretone and X-rayed. The gut of the experimental fish contained a large amount of thorium dioxide, enough to justify a three-plus rating if four plus signs indicate the maximum concentration that we have seen.

The native species tested and certain essential data are shown in Table 1. Specimens of *Nocomis*, *Schilbeodes*, *Ameiurus*, *Umbra*, and *Lepomis* were isolated in 1 liter of water in stoneware jars like those used for the large goldfish. All were kept at 23°–24° C. for the last few days before being X-rayed. They were fed enchytraeid worms daily but were not handled until they were removed to be anesthetized just before being X-rayed. Enough Thorotrast was gently poured into each of the jars to make a concentration of thorium dioxide of 1.5 or 3 per cent (=6 or 12 per cent Thorotrast), in which the respective individuals remained for 1, 5, or 24 hours before X-ray exposures were made.

Individual representatives of the other species tested were isolated by pouring each with its surrounding water into separate finger bowls, 12.5 cm. in diameter. Thorotrast was added to each finger bowl to make a 12 per cent suspension (3 per cent ThO_2) and the fishes were X-rayed 24 hours later. Meantime,

enchytraeid worms were placed in the Thorotrast suspensions as possible food for the fishes, but the *Esox*, *Fundulus*, and *Micropterus* did not eat; in fact, the *Esox* fasted during its whole 4-day stay in the laboratory preceding the X-ray exposure. Feeding or lack of feeding by goldfish or other species made no observable difference in the ingestion of Thorotrast.

The thorium dioxide swallowed by experimental fishes is clearly seen to be in the gut. It was present in most individuals in each of the species tested. No consistent differences were detected between different durations of immersion in the Thorotrast or between the different species, perhaps because the experiments were primarily exploratory rather than analytical. Representative photographs of roentgenograms of different species and their controls are shown in Plate I. In order to avoid the possible effects of handling on water ingestion (Smith, 1930), we used different untreated individuals as controls in place of X-raying the same fish before exposure to Thorotrast.

As in the previous more intensive series of tests with goldfish, no trace of thorium dioxide was observed in gill or oral regions of healthy fishes. In several instances in which fishes were X-rayed after dying in dilute Thorotrast, or when obviously injured by exposure to it, rather large amounts of thorium dioxide were present on the gills. Such collections are shown in both photographs of *Boleosoma* (Fig. 4) in distinct contrast with the living healthy survivor of similar treatment shown in Figure 9. Similar collections may be seen in the upper picture of *Nocomis* (Fig. 7) and in the upper picture of *Fundulus* (Fig. 8). We do not believe any thorium dioxide is shown accumulated in the mouth region of the fish just above the untreated *Fundulus* control. We repeat for emphasis: All individuals showing such collections in mouth or gill regions were ill or had died shortly before being X-rayed. These collections of thorium dioxide on the gills are not species specific except as certain species are more sensitive to the toxic action of Thorotrast than are others. Healthy *Boleosoma* (Fig. 9) showed no clear evidence of opacity in the mouth or on the gills beyond that found in the control. Apparently, either the thorium dioxide or some other constituent of

TABLE 1

NATIVE FISHES EXPOSED TO THOROTRAST AND A SUMMARY OF THEIR LABORATORY TREATMENT

Species	Family	Days in Laboratory	ThO_2 Concentration (Per Cent)	Hours in Thorotrast	No. Treated
Erimyzon sucetta	Catostomidae	4	3	24	1
Nocomis biguttatus	Cyprinidae	14+	1.5	1, 5, 24	3
Ameiurus melas	Ameiuridae	6	1.5	1, 5, 24	4
Schilbeodes insignis	Ameiuridae	14+	1.5	1, 5, 24	3
Umbra limi	Umbridae	4	1.5	1, 5, 24	3
Esox vermiculatus	Esocidae	4	3	24	1
Fundulus diaphanus	Cyprinodontidae	4	3	24	3
Boleosoma nigrum	Percidae	5	3	24	8
Lepomis cyanellus	Centrarchidae	14+	1.5	1, 5, 24	3
Micropterus dolomieu	Centrarchidae	8	3	24	1

Thorotrast is toxic for certain species of fishes. We found no evidence of toxicity after small goldfish had been exposed to a 4 per cent suspension of thorium dioxide for 24 hours, to 2.6 per cent for 72 hours, and to 1.3 per cent for 96 hours; but goldfish are notoriously hardy. All deaths or injuries in the present experiments occurred with exposure of 24 hours or less in a 3 per cent suspension.

DISCUSSION

Roentgenograms of several taxonomically diverse species of small freshwater fishes that had previously been swimming in suspensions of colloidal thorium dioxide give a clean-cut demonstration of its presence in the gut of each species tested. These observations confirm and extend our previous finding (Allee and Frank, 1948) that goldfish ingest such material.

The method of intake is not yet demonstrated with finality. There are two possible explanations. One assumes that colloidal and other small particles are caught on either oral or gill membranes, or both, perhaps concentrated in the mouth, and are swallowed with little accompanying water. We originally thought that some such process might be operating. The other explanation assumes that small particles, like dissolved materials, are swallowed incidentally to the swallowing of water and are concentrated in the gut.

X-ray studies of healthy fishes give no clear evidence suggesting that thorium dioxide is collected in the oral-gill region. Also dissection of goldfish, previously exposed to finely divided carbon, under a binocular dissecting microscope revealed no sign of a collection of carbon particles in the mouth or on the gills, although carbon was readily visible in the gut. Collections of traces of cells of the alga *Chlorella vulgaris* were found in the gill region (Allee and Frank, 1949); these are 0.012 mm. in diameter. Such collections, if made, apparently are not transferred to the gut by ciliary action. Our inspections have not revealed evidence of ciliated oral or branchial epithelium, but we have not made microscopic sections. Krause (1923) did not find oral cilia in *Esox lucius*, and our tests showed that *E. vermiculatus*, like all other species tested, had swallowed thorium dioxide. *Nocomis*, *Fundulus*, and some *Boleosoma* became ill, and some individuals died during 24 hours of exposure to a 3 per cent suspension of this material (12 per cent Thorotrast).

We attempted no extensive tests directed toward determining toxicity limits for any species. Hasler and Faber (1941) tried the effect of injecting Thorotrast as a means of tagging small fishes. They used trout 4–6 cm. long, weighing 2–3 gm. Their experiments were successful when small amounts were injected. One milligram per fish allowed the injected individuals to increase in weight at the same rate as did the controls; 5.2 mg. per fish and larger amounts caused a high percentage of deaths.

This may perhaps be compared with similar toxic reactions following injection of Thorotrast in man (Reeves and Stuck, 1938; Stenstrom and Vigness, 1940); the mode of action of Thorotrast in the gut must be assumed to be different, however. There are no indications that colloidal thorium dioxide is absorbed from the digestive tract, and the rapidity with which toxic symptoms became manifest in a few of our fishes precludes any effect resulting from the radioactivity of thorium. Death in the latter cases may be hastened in the end by adsorption of the thorium dioxide on the gills, perhaps similar to the action of lead salts (Carpenter, 1927); it is pos-

sible that the dextrin contained in the preparation led to the effects described.

Roentgenograms of ill or dead fishes—and of no others—revealed a collection of thorium dioxide on their gills. The mechanisms involved are unknown; apparently, the accumulation occurs when the rate of flow of water past the gills is sufficiently reduced. Conceivably, it could result from a breakdown of hypothetical cleaning and swallowing processes that could operate to keep such areas clear of accumulations in healthy fishes. These processes may involve a lessening or a cessation of the swallowing of water.

Recognizing the possibility of another interpretation, we tend strongly toward regarding the observed accumulations on the gills of sick fishes as a special problem. This means that we interpret all the data at hand as indicating that healthy fresh-water fishes normally swallow water together with suspended or dissolved materials. We have found no real evidence to date against that interpretation.

Information is lacking concerning the rate at which Thorotrast (or water) is engulfed under the conditions of our experiments. Although this problem can easily be attacked and there is obviously a need for such information, research on this point is not a part of the working program of either of the authors. Neither do we plan to study the stimulus situation that causes fresh-water fishes to swallow water. It is well known that they are hypertonic to the water around them and that the elimination of water received through osmotic inflow is a prominent phase of their ecological physiology (Smith, 1932). Marine fishes are hypotonic to their environment and drink sea water in maintaining water balance. Smith (1930) states that handling marine fishes leads to increased water intake.

The fishes that we have tested to date showed no easily observable difference, if any, in accumulation of thorium dioxide in relation to relatively gentle handling in comparison with no handling at all for a few days before Thorotrast was poured into the water around them. The conditions under which they were housed may have been sufficiently disturbing to produce stimulation. They were kept in relatively small containers, either in opaque stoneware jars or, if small enough, in clear-glass finger bowls. No differences in intake of thorium dioxide were observed to be correlated with the type of container. The small size of the vessels led to frequent wall contacts that probably were stimulating. The fishes were kept in a relatively quiet laboratory, but no effort was made to control laboratory traffic.

In contrast with evidence for delayed swallowing of water found by Ellis (1937), three of six goldfish that we had exposed to Thorotrast for only 1 hour before they were X-rayed showed appreciable amounts of thorium dioxide in the gut. Rough inspection of the roentgenograms revealed no constant differences between those in Thorotrast for 1, 5, 6, 72, or 96 hours and no greater variation than occurred among those exposed for 24 hours. There is some evidence that maximum X-ray opacity in the gut of goldfish is attained after 24 hours in Thorotrast suspensions (Allee and Frank, 1948).

Of the fishes with which the present report is directly concerned, the sunfish (*L. cyanellus*), bullhead (*A. melas*), and cyprinid (*N. biguttatus*) had thorium dioxide in the gut after exposure to Thorotrast for 1 hour; the mud minnow (*U. limi*), and mad-tom (*S. insignis*) had none. The darter (*B. nigrum*) had not swallowed Thorotrast until after some 15 hours of exposure to it. These results show that, under the conditions of our

PLATE I

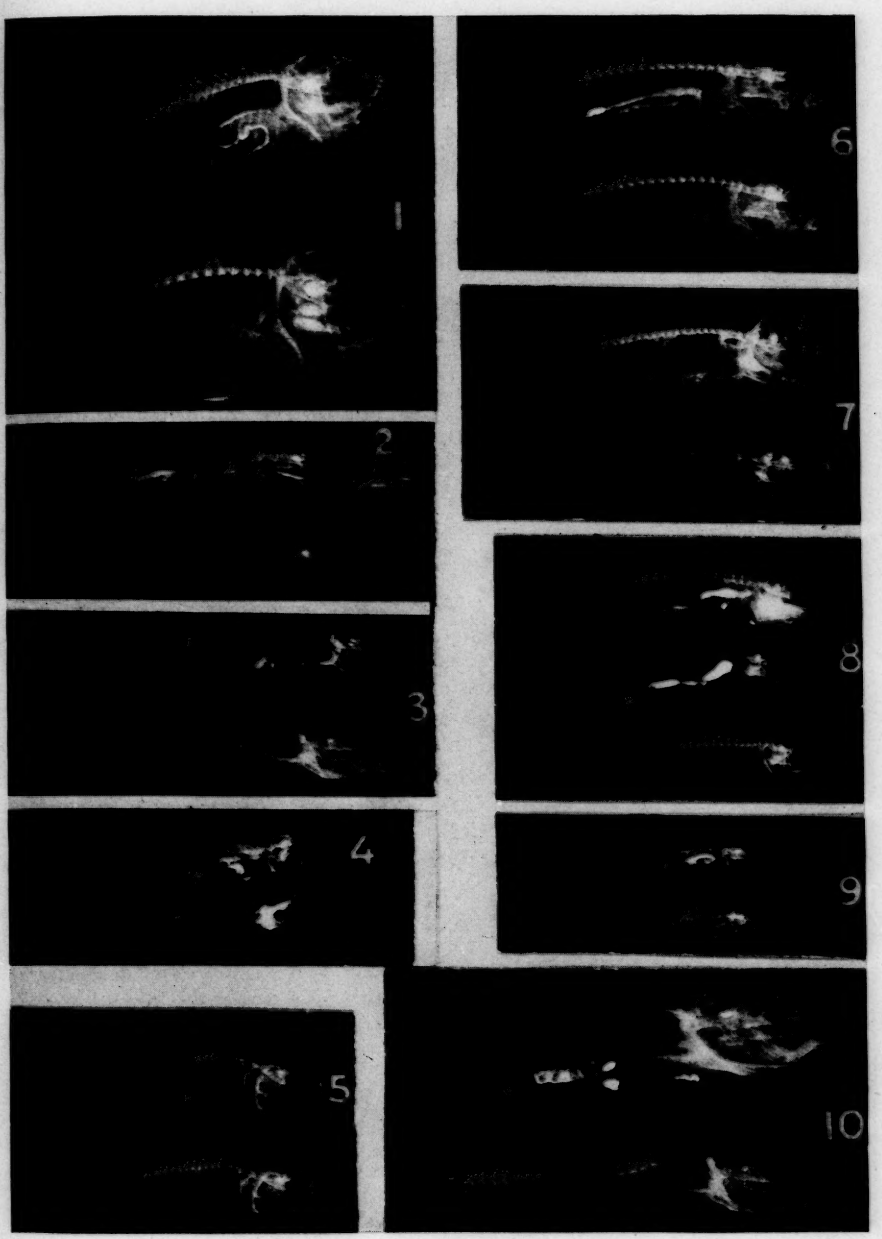
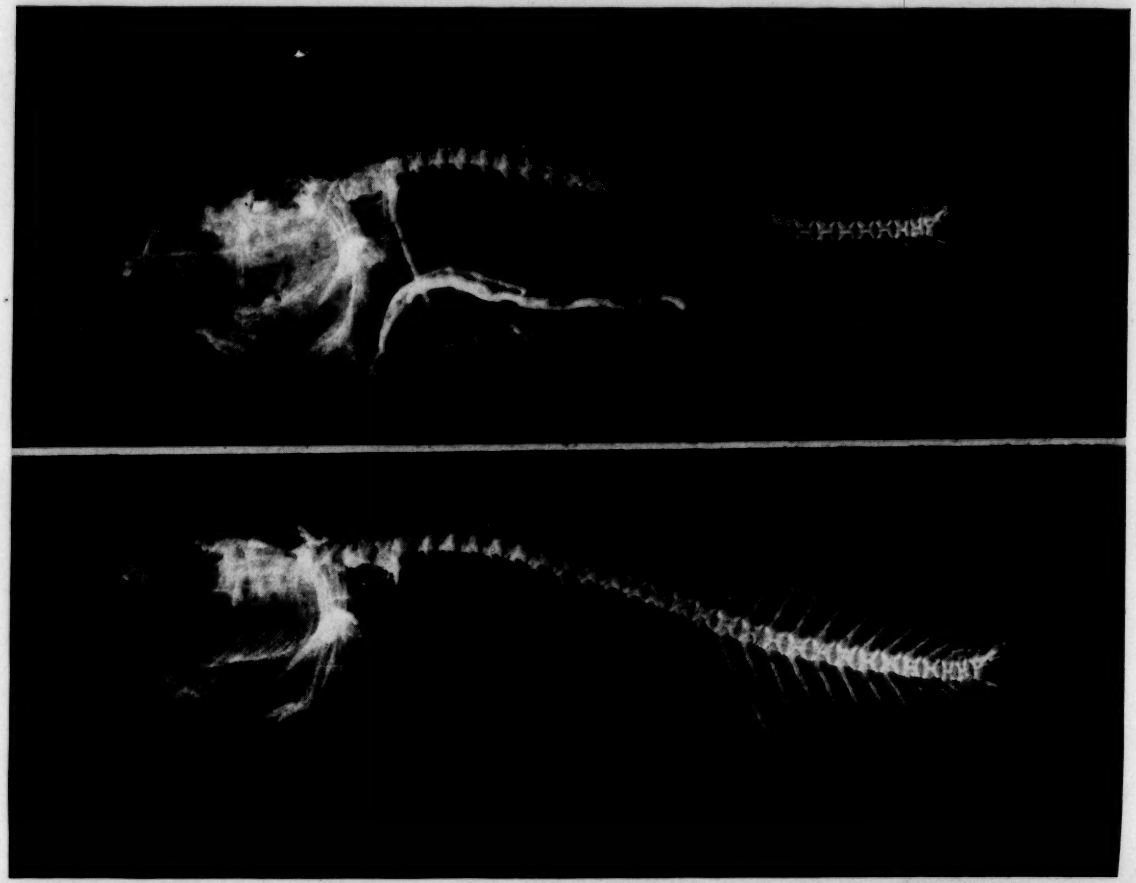


PLATE II



experiments, some fishes swallow this material shortly after its introduction.

The impelling stimulus for our work in this particular field came from the long experience of Allee and a series of associates that regurgitated food particles in homotypically conditioned water have supplementary food value for goldfish, although they are not the sole cause of increased growth or better survival of assay fish isolated in a conditioned medium. The nutritional situation under such circumstances is summarized and extended by Allee and Frank (1949). The method of intake of small food particles is amply explained by the possibilities growing out of the assumption that some are swallowed in the process of swallowing the containing water.

SUMMARY

1. Roentgenograms demonstrated that all families of fresh-water fishes tested engulf colloidal thorium dioxide during an exposure of 24 hours or less. The families tried were: Catostomidae, Cyprinidae, Ameiuridae, Umbridae, Esocidae, Cyprinodontidae, Percidae, and Centrarchidae.

2. Swallowing of water with the contained suspension is the most plausible explanation of the method of intake.

LITERATURE CITED

ALLEE, W. C., and FRANK, PETER. 1948. Ingestion of colloidal material and water by goldfish. Physiol. Zoöl., 21:381–91.

———. 1949. The utilization of minute food particles by goldfish. *Ibid.*, 22:346–58.

CARPENTER, KATHLEEN E. 1927. The lethal action of soluble metallic salts on fishes. Brit. Jour. Exper. Biol., 4:378–90.

ELLIS, M. M. 1937. Detection and measurement of stream pollution. Bull. Bur. Fisheries, 48:365–437.

HASLER, ARTHUR D., and FABER, WILLIAM M. 1941. A tagging method for small fish. Copeia, 1941, No. 3, pp. 162–65.

KRAUSE, RUDOLF. 1923. Mikroskopische Anatomie der Wirbeltiere in Einzeldarstellungen. IV. Teleostier, Plagiostomen, Zyklostomen und Leptokardier. Berlin: Walter de Gruyter.

KROGH, AUGUST. 1939. Osmotic regulation in aquatic animals. Cambridge: At the University Press.

REEVES, D. L., and STUCK, R. M. 1938. Clinical and experimental results with Thorotrast. Medicine, 17:37–73.

SCHUSTER-WOLDAN, E. 1936. Röntgenologische Darmuntersuchungen an *Cyprinus carp.* und *Tinca vulg.*, als Beitrag zur Frage der Bedeutung des Geruchs- und Gesichtssinnes vom Fisch bei der Nahrungssuche. Zeitschr. f. Fisch., 34:245–47.

SMITH, HOMER W. 1930. The absorption and excretion of water and salts by marine teleosts. Amer. Jour. Physiol., 93:480–505.

———. 1932. Water regulation and its evolution in the fishes. Quart. Rev. Biol., 7:1–16.

STENSTROM, W., and VIGNESS, I. 1940. Elimination of radioactive elements in patients who have received Thorotrast intravenously. Proc. Soc. Exper. Biol. and Med., 44:18–22.

PLATE I

With healthy fishes the untreated control individual is placed at the bottom of each figure.

FIG. 1.—*Lepomis cyanellus*, green sunfish.

FIG. 2.—*Esox vermiculatus*, grass pickerel.

FIG. 3.—*Schilbeodes insignis*, mad-tom.

FIG. 4.—*Boleosoma nigrum*, Johnny darter; ill or dead.

FIG. 5.—*Erimyzon sucetta kennerlii*, chub sucker.

FIG. 6.—*Umbra limi*, mud minnow.

FIG. 7.—*Nocomis biguttatus*, chub.

FIG. 8.—*Fundulus diaphanus menona*, top minnow.

FIG. 9.—*Boleosoma nigrum*, Johnny darter; healthy.

FIG. 10.—*Ameiurus melas melas*, black bullhead.

PLATE II

Carassius auratus, goldfish, large

DIFFERENTIAL MODIFICATION OF SAND-DOLLAR DEVELOPMENT IN RELATION TO TEMPERATURE

C. M. CHILD
School of Biological Sciences, Stanford University

THE evidence from earlier studies of the modification of development by external agents in echinoderms and various other forms has indicated that many different agents produce essentially similar modifications, i.e., the modifications are, to a high degree, nonspecific in character for different agents. They are, however, regionally graded in degree in relation to the physiological axes of polarity and ventrodorsality or dorsoventrality. In other words, they are differential and suggest that the characteristics of physiological pattern which determine them are primarily quantitative gradients involving the basic metabolic activities of the organism concerned. In consequence of the higher susceptibility or sensitivity of the higher gradient levels to the agents, differential inhibitions of development tend to decrease, and may even obliterate, physiological polarity, ventrodorsality, and the right-left coelomic asymmetry in echinoderms. On the other hand, differential tolerance and acclimation, i.e., acquirement of increased tolerance to the less extreme effects of inhibiting agents, and differential recovery after return to natural conditions may determine differential developmental modifications opposite in character to the differential inhibitions. These increase the differentials and steepen the gradients because the higher, more active gradient levels are more tolerant of slight effects of inhibiting agents, acquire increased tolerance, and recover more rapidly and more completely than less active gradient levels, provided that the inhibiting action has not been too severe. Direct differential acceleration of development has also been obtained in some cases with increase of gradient differentials and developmental modifications opposed in direction to the differential inhibitions. These relations of early developmental pattern to early gradient pattern, or at least some of them, have been obtained not only in echinoderms but in many other forms.[1]

It has long been known that development is accelerated by a rise in external temperature up to a certain limit, i.e., to the threshold of injury, and is retarded or even stopped by a decrease in external temperature. So far as the writer is aware, however, no definite, positive evidence has been presented to show that differential modification of development related to gradient pattern may be brought about by difference in external temperature during early developmental stages.

During the summer of 1948, eggs of the sand dollar, *Dendraster excentricus*, were kept during development at two different temperatures, differing by approximately 10° C. (12° and 22°–24° C.). Differences in the two lots of plutei at the two temperatures were clearly evident, even on cursory microscopic examination. In general, the angle of di-

[1] See Child, *Patterns and Problems of Development* (Chicago: University of Chicago Press, 1941), chaps. v–vii and literature cited there.

vergence of the aboral arms was greater, the oral lobe was larger and, more particularly, wider, and the angle of divergence of the oral arms at its tip larger in the lots at high, than in those at low, temperatures. Measurements of the angles of the aboral and oral arms confirmed the observations. During the summer of 1949, additional data were obtained, confirming the earlier data and bringing to light some further points of interest. This paper is concerned with these data and the question of their significance.

MATERIAL AND METHODS

Three lots of *Dendraster* eggs were used, one lot in 1948, two at different times in 1949. All three lots were fertilized at the Hopkins Marine Station, Pacific Grove, and brought to Palo Alto with almost no change of water temperature (about 18°). The eggs were at the 4–8-cell stage in 1948 and the first lot of 1949, and at a slightly later stage in the second lot of 1949, when they were placed in the different temperatures.[2] Water temperature was not changed suddenly but was gradually brought to the external temperature. Material was kept in covered finger bowls containing approximately 250 ml., four to six bowls at each temperature in each of the three lots of eggs, some thirty-five bowls in all. Numbers of embryos in each bowl were believed to be sufficiently small to avoid effects of crowding, but at the highest temperature used in 1949 there was evidence of slight effects of crowding in most of the containers, as will appear. Eggs of two females were used in each of the three lots, were fertilized separately by sperm from one or two males but, after it was ascertained that fertilization was virtually 100 per cent, were mixed. The low-temperature material was kept at 12° C. in a constant-temperature room in 1948 and 1949. Exact control of the higher temperature was not practicable. In 1948 it was that of a laboratory room, with a range of approximately 22°–24° C. during the developmental period. In 1949 two high temperatures were used, that of a basement room at 20°–22° C. and that of a laboratory room at 22°–25° C. during development.

For determining arm angles, a number of plutei in approximately 1 ml. of water on a large depression slide were immobilized by sea-water chloretone or were killed by formalin. The latter was more satisfactory than chloretone because slight movement usually continued for a considerable time in rather high concentrations of chloretone. Material was also kept in formalin for further examination. The anesthetized or killed plutei lie in various positions, the great majority either with the aboral arms or the oral lobe below, more frequently in the latter position because the oral lobe is heavier. Some of these are tipped slightly to one side, so that the arms appear not quite symmetrically, but large numbers lie symmetrically. Comparatively few lie almost or entirely on one side, and still fewer lie on the tips of the oral lobe and aboral arms.

For determination of arm angles, the arms or lines along their axes were drawn by means of a camera lucida only of those individuals which happened to lie so that the arms and oral lobe appeared symmetrically, as in most of the following figures. In fully developed plutei of the usual forms the planes of oral lobe and aboral arms are so nearly parallel that individuals with arms above or below can be used for determination of angles. However, in plutei with large aboral-arm angles the planes of oral lobe

[2] For facilities provided at the Hopkins Station and to Dr. Olin Rulon and Dr. A. R. Moore for sharing material, the writer is grateful.

and aboral arms often diverge more, so that in animals lying on the oral lobe the aboral arms appear somewhat foreshortened and the angles larger than they actually are. In such animals the arm angles are drawn only with aboral arms below and flat on the glass. If the entire length of the arms is in the same focal plane, the individual is available for determination of the arm angle. The oral arms are much shorter, and their angles can usually be drawn with oral lobe above or below. After arm angles of animals in proper positions or position are drawn in one field, the slide is moved sufficiently to provide another field, care being taken to avoid repeated drawing from the same field. The question of the adequacy of this method of sampling is considered in the discussion.

Camera drawings of aboral-arm angles of fully developed plutei were made in this way in a series of 100 each for the two temperatures in 1948 and in two series of 100 each for 12° and 20°–22° C. in 1949. For comparison with aboral-arm angles of fully developed plutei, a series of one hundred each for the same two temperatures was drawn of the aboral-arm angles of early plutei at stages when the arms had become long enough to permit determination of direction of outgrowth. Three series of camera drawings of oral-arm angles of fifty each for the same low and high temperatures were also made, two from the 1948 material, one from that of 1949. On these camera lucida drawings the angles were measured with a protractor and a straight edge. Repeated measurements of the same angles showed an accuracy to approximately 2 degrees. From the measurements in angular degrees, averages were determined for each twenty-five and total averages for each one hundred of aboral arms and each fifty of oral arms. In addition to the averages, frequencies were determined and plotted for each temperature pair, the low- and high-temperature frequencies of each temperature pair appearing in the same graph. In all graphs, ordinates are numbers of individuals, abscissae are classes in angular degrees.

Provisional graphs were plotted with classes of 4, 5, 8, and 10 angular degrees. The plots from classes of 4 and 5 degrees did not differ greatly but in most cases showed great irregularities and were sometimes without any clearly indicated mode, presumably because of the relatively small numbers of angles in each lot. Plots from classes of 8 degrees were not very different from those based on 10-degree classes; as might be expected, both these plots showed much less irregularity than those from smaller classes.

It is of some interest to discover, so far as the frequency data permit, how closely modes and average angles agree. However, for the present purpose, modes are regarded as less important than the differences in range of the frequencies at the different temperatures. With all the frequency classes used in the provisional graphs, the differences are in the same direction but, of course, differ in magnitude with size of class in graphs plotted on the same scale. Frequency classes of 10 angular degrees were finally chosen as most satisfactory for the purpose of the paper. These classes are perhaps rather large but do show clearly the relations of low- and high-temperature frequencies, and this is regarded as the point of chief importance. However, in two cases graphs plotted from 5-degree classes are also presented for comparison with the 10-degree graphs of the same lots.

Tables and graphs are supplemented by figures indicating the ranges of variation in form and proportions. All figures are from camera drawings at the same

magnification and consequently represent size and other differences on the same scale.

DEVELOPMENT AT THE DIFFERENT TEMPERATURES

At the two higher temperatures, 20°–22° and 22°–25° C., used in 1949, the plutei attained full size and apparently complete differentiation in approximately 48 hours or slightly less from the time of exposure to the temperature. At 12° this stage was attained only after 96 hours or slightly longer. Although there is a wide range of variation in form and proportions of both early and full-grown plutei at each temperature, differences which are, in general, characteristic for the two temperatures appear. If development at one temperature is regarded as the norm, development at the other or others may be regarded as differentially modified. However, as will appear, some individuals are essentially similar at low and high temperatures: the differences at the different temperatures are, to a large extent, differences in frequencies of the various forms. At both 12° and 20°–22°, virtually 100 per cent of the eggs developed into large active plutei in most of the containers. As usual in lots of echinoderm material, examination of large numbers of individuals showed a few—less than 1 per cent—not fully developed or clearly abnormal in one way or another. In the lots developing at 20°–22° this was also the case except in two containers, in which certain differential modifications of form appeared in 10–20 per cent of the individuals, the remainder being well-developed plutei. At the highest temperature—22° to 25°—similar, but often more extreme, differential modifications ranged from 5–10 per cent up to 80–90 per cent in different containers; but in all these lots there were also large, well-developed plutei, some even further developed than is usual without food. The modified forms which could not be regarded as within the normal range were all of the same general character but differed in degree of modification.

The containers were thoroughly washed in both fresh and salt water before being used, and contamination from earlier use appears highly improbable. Moreover, the presence in all these containers of large, fully developed plutei with the high-temperature characteristics indicates that there was no general contamination sufficient to determine the differentially modified forms. These forms are regarded as of considerable interest in relation to the usual high-temperature forms, and the question of the conditions determining them is considered in a later section of this paper in connection with descriptions and figures of the forms.

ANGLES OF DIVERGENCE OF ABORAL ARMS IN EARLY PLUTEI AT LOW AND HIGH TEMPERATURES

Incidental observations and measurements indicated that the arm angles are, in general, larger in early than in fully developed plutei. For this reason angles were determined in early plutei as soon as the arms were long enough to permit determination of direction of outgrowth. Averages for lots of 25 each and for the entire 100 at low and high temperatures appear in Table 1. Separation into lots of 25 is merely for the purpose of providing some indication of the range of variation of averages. At the low temperature the early pluteus stage permitting determination of angles was reached 51 hours after the containers were brought into the low-temperature room at 12° C. At the high temperature approximately the

same stage was reached 26 hours after the beginning of exposure to 20°–22° C.

It is evident from Table 1 that the variations are not great in the four lots of 25 each which make up the 100 at each temperature, but the arm angles at high temperature average 9.4 angular degrees larger than at low temperature. The graph (Fig. 1) shows the frequency distributions of the measured aboral-arm angles of the early plutei in the low- and high-temperature lots of 100 each. The low-temperature frequencies are shown in unbroken line, the high-temperature frequencies in broken line. It is evident at once that the modes indicated on the basis of the 10-degree classes are in the same class for the two temperatures but higher (33 individuals) at the low than at the high temperature (24 individuals). Incidentally, the low-temperature modal class, 51–60 degrees, and the low-temperature total average in Table 1 (56.5 degrees) represent approximately the same sizes of angles. At the high temperature the frequency at 61–70 degrees differs by only one individual from the mode at 51–60 degrees. This suggests that a larger number of measurements might shift the mode to the higher class and bring it nearer the total average (65) in Table 1. Of greater importance than the modes is the difference in frequency distribution and range of arm angles at the two temperatures. At the low temperature, 8 individuals have arm angles in the class 31–40 degrees; at the high temperature only 1 is in this class. Low-temperature frequencies are higher for all angles up to and including 61–70 degrees than high-temperature frequencies. Above 61–70 degrees the effect of high temperature appears both in higher frequencies and in arm angles larger than any at low temperature. At low temperature there are 3 individuals in the class, 81–90 degrees; at high temperature, 7. Also there are no angles above 81–90 degrees at low temperature, while at high temperature 10 individuals have larger angles, 1 of them 30 degrees larger. The low-temperature plot is almost symmetrical; the high-temperature plot is shifted to the right as a whole and distinctly unsymmetrical. Up to and including the class 61–70 degrees, the total number of individuals is 90 at low temperature, 66 at high. In higher classes it is 10 at low temperature, 34 at high.

TABLE 1

AVERAGES OF ABORAL-ARM ANGLES IN EARLY PLUTEI AT LOW AND HIGH TEMPERATURES

Temp.	Lots of 25 and Totals
Low	59.3
	56.7
	56
	54
Total	56.5
High	65.6
	64
	67.4
	66.6
Total	65.9

DIFFERENCES IN FORM AND PROPORTIONS OF EARLY PLUTEI IN RELATION TO TEMPERATURE

Differences not only in arm angles but in form and proportions of early plutei are evident to the eye at once. Figures 2–7 indicate approximately the range of difference in arm angles measured in early plutei at 12° C. It is from less than 40 degrees in Figure 2 to more than 80 degrees in Figure 7. The oral lobe in these low-temperature early plutei is merely an outgrowth of the apical polar region, decreasing from the median region laterally and without any indication of the oral arms (Figs. 2–7).

Figures 8 and 9, early plutei seen from the side, show the angular relations between the oral lobe at the right and aboral arms at the left, with different degrees of arm development relative to the oral lobe in the two figures. The arm is, of course, foreshortened in this view. The planes of oral lobe and aboral arms diverge only slightly in Figures 8 and 9, and this is characteristic of early plutei at low temperature. The deeply concave region in these side views, extending from the tip of the oral lobe to the aboral arm, is commonly regarded as ventral. Low-temperature early plutei, supported on the tips of the oral lobe and aboral arms and seen from above in almost dorsal view, appear as in Figures 10 and 11, with oral lobe at the top of the figure. In this position oral lobe and arms are foreshortened, and the arm angles are not visible.

Figures 12–20 show the forms and range of arm angles in early plutei of the high-temperature lot, and the side views (Figs. 21–23) show the variations in divergence of the planes of oral lobe and aboral arms. This is slight (Fig. 21) in the individuals with the smaller aboral-arm angles, such as Figures 12–14. With larger aboral-arm angles, greater divergence between oral lobe and arms becomes more frequent (Figs. 22 and 23), and arm angles must be determined in individuals lying with aboral arms below (Figs. 17–20). In animals lying on the oral lobe the arms are foreshortened, and the arm angles appear larger than they actually are, as becomes evident when these individuals are

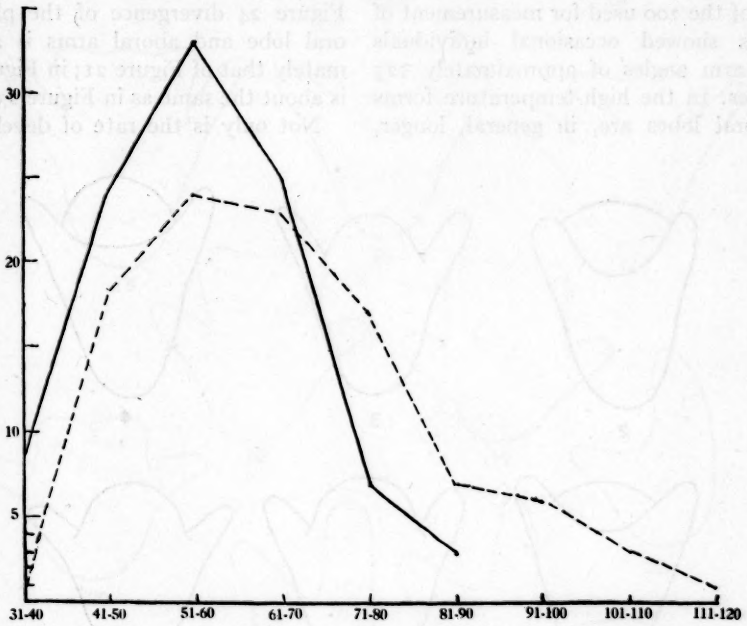
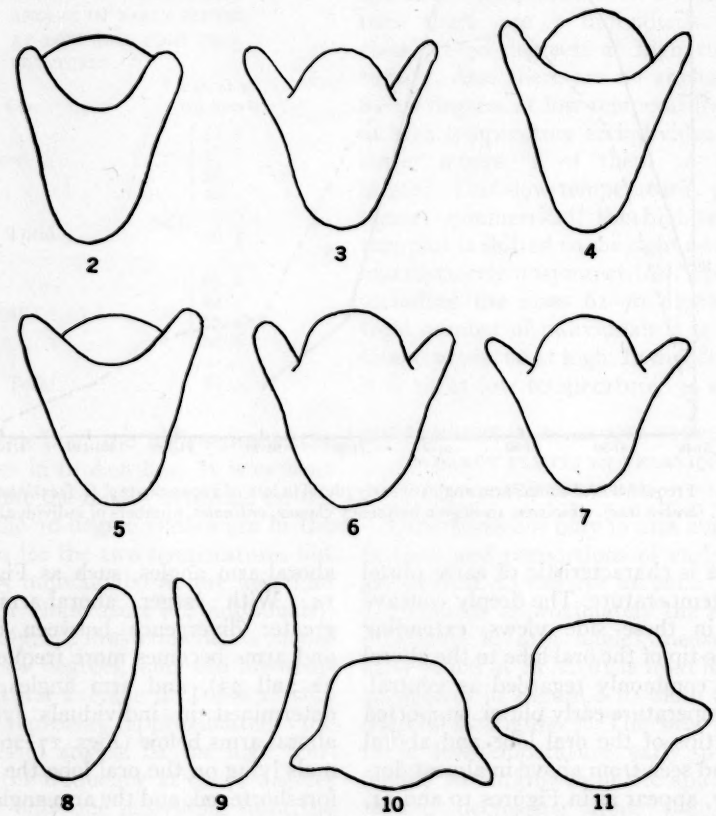


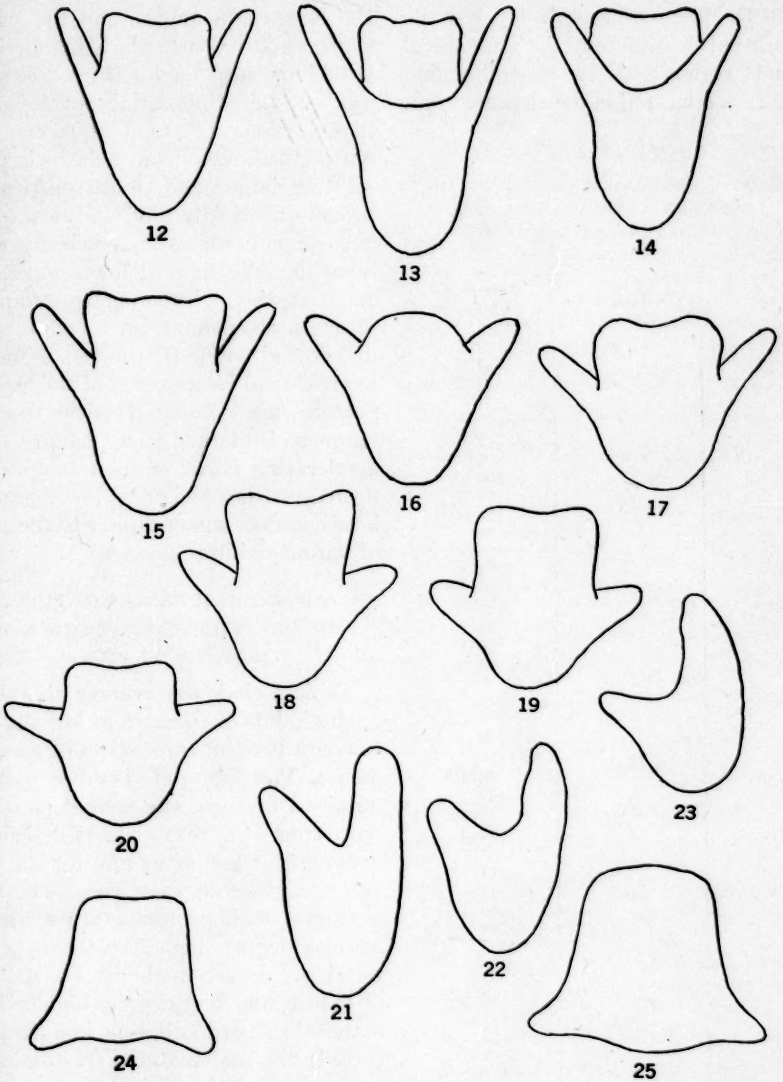
FIG. 1.—Frequencies of aboral-arm angles of early plutei in lots of 100 each at 12° C. (*continuous line*) and 20°–22° C. (*broken line*). *Abscissae*, 10-degree frequency classes; *ordinates*, numbers of individuals.

turned over. Figures 15–20 show early plutei with arm angles of approximately 75–125 degrees. Only 10 of the low-temperature early plutei had arm angles as large as those of Figures 15 and 16, and no larger angles were observed; in the high-temperature lot there were 23 individuals with arm angles larger than these. Moreover, examination of high-temperature early plutei which were not part of the 100 used for measurement of angles showed occasional individuals with arm angles of approximately 125 degrees. In the high-temperature forms the oral lobes are, in general, longer, wider, and more advanced in development and show early developmental stages of the oral arms (Figs. 12–15, 17–20). In a few individuals the lobe is still merely an outgrowth of the apical region of earlier stages, rounded mediolaterally as in the low-temperature forms (Fig. 16). Figures 24 and 25 indicate range of size and proportions of high-temperature early plutei in almost dorsal view. In Figure 24 divergence of the planes of oral lobe and aboral arms is approximately that of Figure 21; in Figure 25 it is about the same as in Figure 23.

Not only is the rate of development



FIGS. 2–11.—Forms and aboral-arm angles of early plutei at 12° C. Figs. 2, 4, and 5, arms above; Figs. 3, 6, and 7, arms below. Figs. 8 and 9, on side; Figs. 10 and 11, on tips of oral lobe and aboral arms.



FIGS. 12–25.—Forms and aboral-arm angles of early plutei at 20°–22° C. Figs. 12 and 15–20, arms below; Figs. 13 and 14, arms above. Figs. 21–23, on side. Figs. 24 and 25, on tips of oral lobe and arms.

of the high-temperature forms double that of the low-temperature lot, but the acceleration is differential. The apical (animal) region and the ventral region are more accelerated in development and relatively or absolutely larger at the high than at the low temperature. The apical region is the high end of the polar component of the gradient pattern, the ventral region the high end of the ventrodorsal component. The wider oral lobes, the larger arm angles, and the smaller, shorter bodies, particularly in individuals with large arm angles (Figs. 18–20, 22, 23), are all differential developmental modifications, i.e., ventral, as compared with dorsal, regions are relatively or absolutely larger and, more particularly, broader mediolaterally, and in the more extreme individuals there is less development dorsally than at low temperature. In short, the high-temperature forms indicate a steepening, an increase in the differentials of both the polar and the ventrodorsal components of the gradient pattern, as compared with the low-temperature forms. Susceptibility to the accelerating effect of high temperature decreases from higher to lower gradient levels, as does susceptibility to the action of various inhibiting agents.[3]

TABLE 2

AVERAGE OF ABORAL-ARM ANGLES IN FULLY DEVELOPED PLUTEI AT LOW AND HIGH TEMPERATURES

Year	Temp.	Lots of 25 and Totals
1948	Low	53.6 58.1 57.8 61
		57.6
	High	65.1 61.3 61.4 64.8
		63.1
1949*A*	Low	48.8 44 47.1 43.3
		45.8
	High	49.6 50.3 55.4 58
		53.3
1949*B*	Low	42.9 40.4 43.4 41.5
		2
	High	48.3 54 54.2 52.6
		52.3

ABORAL-ARM ANGLES OF FULLY DEVELOPED PLUTEI AT LOW AND HIGH TEMPERATURES

Table 2 gives the average aboral-arm angles in lots of 100 each at low and high temperatures for three series of measurements. The first pair is from material obtained in 1948, the second and third pairs from 1949 material. As in Table 1, averages for lots of 25 and for the total 100 are given in each case. The total averages at high temperature are 5.5 angular degrees higher in the 1948 material, 7.5 degrees higher in the material of 1949*A*, and 10.3 degrees higher in the material of 1949*B* than at low temperature. In the 1948 material the total averages at both low and high temperatures are approximately 10–15 degrees higher than the corresponding averages in the two pairs in 1949 and almost the same as the averages for early plutei of 1949. Arm angles of early plutei were not measured

[3] Child, *op. cit.*, chap. vi and literature cited there.

in 1948. In the pair of 1949*B* the total average at low temperature is 14.5 degrees less, that at high temperature 13.6 degrees less, than the corresponding averages of the early plutei in Table 1, which were from the same material. These differences confirm a difference directly visible to the eye in early and fully developed plutei. There is a distinct decrease in aboral-arm angles from their earliest stages to their full development and apparently also later. The differences in arm angles between the 1948 and 1949 material suggest a difference in the physiological condition of the material of the two breeding seasons. The nature of the difference can only be surmised, and the conditions determining it are completely unknown, but the suggestion seems permissible that the 1948 material may have been at slightly higher metabolic levels than that of 1949.

The frequencies are graphed in Figures 26–29. Since the material consisted of three lots of eggs, one obtained in 1948 and two at different times in 1949, it has seemed advisable to present a graph for the temperature pair of each lot of material rather than a single graph for all pairs. Figure 26, the graph of aboral-arm angles in the 1948 material, shows a flat low-temperature mode from 31 to 50 degrees, 10–20 degrees below the high-temperature mode and below the total average 57.6 in Table 2. A second mode appears at 71–80 degrees, but this may be merely an irregularity resulting from the small number of angles measured. The high-temperature mode at 51–60 degrees is also below the high-tempera-

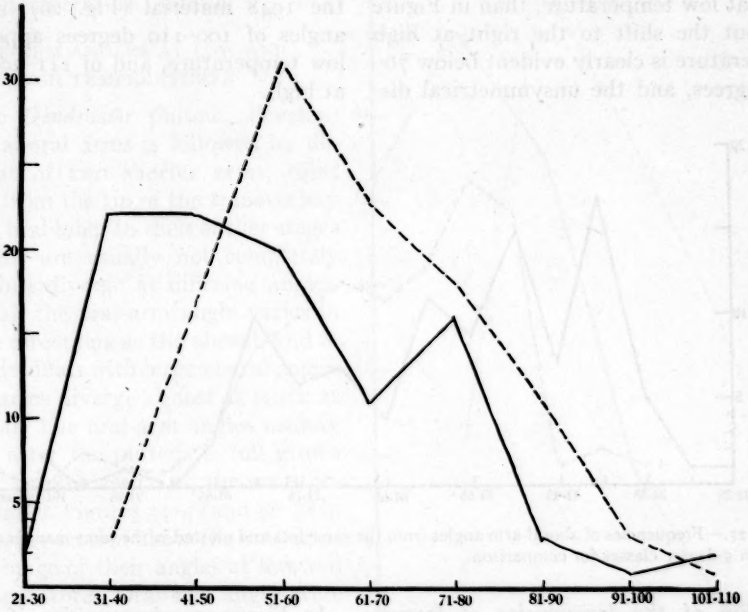


FIG. 26.—Frequencies of aboral-arm angles of fully developed plutei in lots of 100 each at 12° C. (*continuous line*) and 22°–24° C. (*broken line*). From 1948 material. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

ture average, 63.1 (Table 2). In this temperature pair the chief shift to the right in the high-temperature frequencies is in the smaller angles at the left, but the shift is also evident in the large angles at the right.

For comparison with Figure 26 the frequencies of the same pair are plotted from 5-degree classes in Figure 27. Irregularities are much greater, particularly at low temperature, than in Figure 26; but the shift to the right at high temperature is clearly evident below 76–80 degrees, and the unsymmetrical distribution of the frequencies of larger angles at both temperatures is also present, though in a somewhat different relation from that in Figure 26.

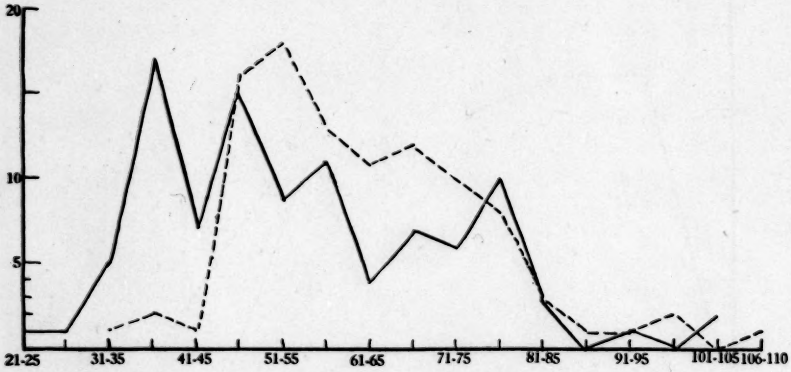


FIG. 27.—Frequencies of aboral-arm angles from the same lots and plotted in the same manner as Fig. 26, but from 5-degree classes for comparison.

Aboral-arm frequencies of the temperature pair from the first material of 1949 (1949*A* of Table 2) are plotted in Figure 28. The high-temperature frequencies are far to the right of the low. At the low temperature, 26 plutei are in the class 21–30 degrees; at the high temperature none are in this class, and only 11 in the class 31–40 degrees. The low-temperature mode at 31–40 degrees with 35 individuals and the high-temperature mode at 41–50 degrees with 38 individuals are both slightly below the total averages in Table 2. The frequencies at the right of the indicated modes, i.e., for the larger angles, are much greater at high than at low temperature except in the highest class, 71–80 degrees. No angles larger than 71–80 degrees appeared at either temperature in the 100 measured, a considerable difference from the 1948 material (Fig. 26), in which angles of 100–110 degrees appeared at low temperature, and of 111–20 degrees at high.

In Figure 29, frequencies of aboral-arm angles are plotted from material obtained 18 days later than that of Figure 28, but still at or near the height of the breeding season (1949*B*, Table 2). The high-temperature frequencies are lower at the left and higher at the right than the low-temperature frequencies, but the indicated modes are in the same class, 41–50 degrees. When plotted from 5-degree classes, the low-temperature mode is in the class 41–45 degrees, the high-temperature mode at 46–50 degrees, but there is a difference of only one individual in the two classes at both tem-

peratures. Here also the modes are below the total averages for 1949*B* in Table 2.

In all the graphs of aboral arms of fully developed plutei the high-temperature frequencies are shifted to the right as compared with low-temperature frequencies, i.e., larger angles appear more frequently at high, than at low, temperature. The measurements of these angles confirm direct observation of living low- and high-temperature material.

ORAL-ARM ANGLES AT LOW AND HIGH TEMPERATURES

In the *Dendraster* pluteus, development of aboral arms is followed by development of two shorter arms, right and left, from the tip of the transversely flattened oral lobe. In their earlier stages these arms are usually not completely parallel but diverge at differing angles. In general, the oral-arm angle varies in the same directions as the aboral, and in some individuals with large aboral angles the oral arms diverge almost as much as the aboral. The oral-arm angles usually decrease after the pluteus is full grown and may become zero, i.e., the arms become parallel. Figures 34–42 and 48–58 in the following section show the oral arms and the range of their angles at low and high temperatures. Oral-arm angles were measured in two lots of 25 each in each of two temperature pairs from different containers of the 1948 material and in two lots of 25 each in a single temperature pair from 1949 material—in all, three lots of 50 each at low and at high temperature. Averages for each 25 and for the totals of 50 appear in Table 3. The total averages are, respectively, 15.7, 8.2, and 9.6 angular degrees greater at the high temperature than at the low. Angular frequencies of the measurements are graphed in Figures 30–33. Here also it has seemed advisable to graph the frequencies of the three temperature pairs separately because of the differences in distribution and in a graph plotted from 5-degree classes for comparison. In all these graphs the frequencies are shifted to the right at high, as compared with low, temperature; also the modal frequencies are lower at high than at low

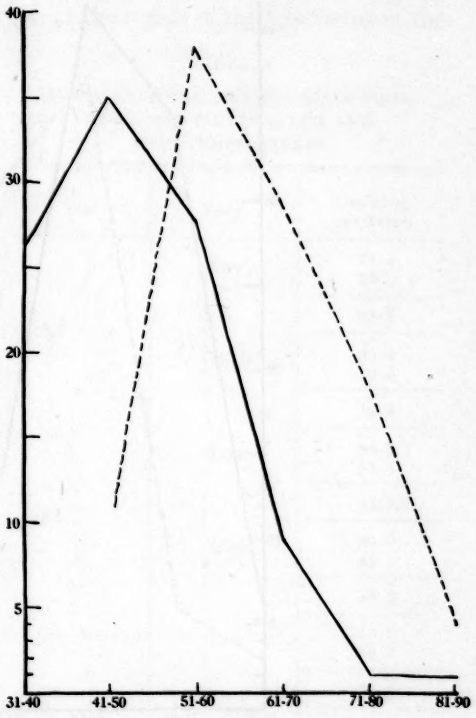


FIG. 28.—Frequencies of aboral-arm angles of fully developed plutei in lots of 100 each at 12° C. (*continuous line*) and 20°–22° C. (*broken line*). From earlier 1949 material. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

temperature. In Figure 32 the frequencies in the class 0–9 differ from the indicated mode by only 2. An additional point of interest as regards the material of Figure 32, but which is not shown in the graph, is that at low temperature the oral-arm angle in the class 0–9 degrees is zero in 13 of the 14 individuals in

this class, while at high temperature the angle is zero in only 5 of the 11 individuals in the class. Figure 33, from the same temperature pair as Figure 32, is plotted from 5-degree classes. In spite of the irregularities in this graph, the higher frequencies of the larger angles at high

a very general comparison between the modal frequencies and the total averages in Table 3. As regards modal frequencies, the graphs of oral-arm angles have less significance than the graphs of aboral arms, but the shift to the right of high-, as compared with low-, temperature fre-

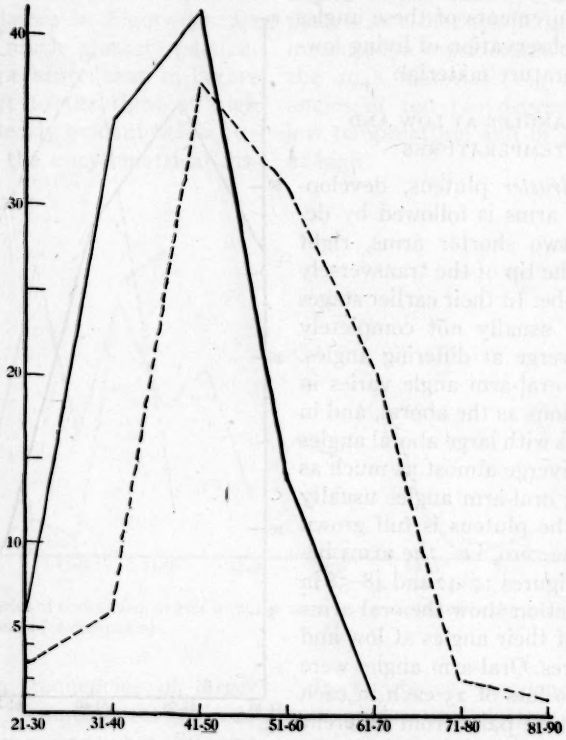


FIG. 29.—Frequencies of aboral-arm angles of fully developed plutei in lots of 100 each at 12° C. (*continuous line*) and 20°–22° C. (*broken line*). From later 1949 material. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

temperature are clearly evident. In all these graphs of oral-arm angles not only is there a shift of frequencies to the right at high temperature, but there are larger angles at high temperature than any at low temperature, particularly in Figures 30 and 32. The 10-degree classes are too large and numbers of angles measured too small to permit anything more than

quencies appears sufficiently definite to be highly significant and is in the same direction as in the graphs of aboral-arm frequencies. Also the average angles of both aboral and oral arms are larger at high temperature than at low. In these respects the data from measurements of aboral- and oral-arm angles are in full agreement.

FORMS AND PROPORTIONS OF FULLY DEVELOPED PLUTEI IN RELATION TO TEMPERATURE

The range of variation observed in form, proportions, and aboral- and oral-arm angles in fully developed unfed plutei at 12° C. from early stages is indicated in Figures 34–47. No more extreme variations were observed in examinations of large numbers of plutei. Forms resembling Figure 34 occur rarely; only 4 such individuals have been seen in the low-temperature material, all in 1949, and only 1 happened to be included in the determination of arm angles. The aboral-arm angle in Figure 34 is very small, the oral arms are parallel, the oral lobe is long but narrow and decreases slightly in width from base to tip. In Figure 35 the aboral-arm angle is somewhat larger, but the oral arms are approximately parallel, and the oral lobe decreases in width from base to tip, as in Figure 34. Figures 36–40, with aboral-arm angles ranging from approximately 35 to 55 degrees, represent the range of the greater low-temperature frequencies in the graphs (Figs. 26–29). The oral arms are parallel or diverge slightly, the oral lobe is wider than in Figures 34 and 35, and there is distinct decrease in width from base to tip only in Figure 37. Figures 41–44, with still larger aboral-arm angles—in Figure 44 more than 100 degrees—are forms which appear much less frequently than those of Figures 36–40, progressively less frequently, with few exceptions, as aboral-arm angles increase. Figures 43 and 44 represent the largest aboral-arm angles observed in all lots of 1948 and 1949. Only 2 plutei like Figure 44 were found in low-temperature lots of 1948, and in 1949 material the largest angles at low temperature were slightly smaller than in Figure 43. As the averages in Table 2 and Figures 26–29 show, aboral-arm angles were somewhat larger in 1948 than in 1949. Figures 34–44 also show that oral lobes are usually larger, increases in width being particularly conspicuous, and oral-arm angles are also larger in plutei with larger aboral-arm angles. Figures 41–44 are particularly good examples. In Figures 34–42 the region of the lobe between the oral arms is deeply concave in outline. In the widest lobes the apical concavity of the lobe is slight, or there is often a slight median elevation (Figs. 43 and 44), i.e., the median region undergoes relatively more growth than in those with smaller lobes and angles. Figures 45–47 are outlines in side view of low-temperature plutei with the oral lobe at

TABLE 3

AVERAGES OF ORAL-ARM ANGLES OF FULLY DEVELOPED PLUTEI AT LOW AND HIGH TEMPERATURES

Year	Temp.	Lots of 25 and Totals
1948*A*	Low	21.4
		26
		23.7
	High	39.4
		39.5
		39.4
1948*B*	Low	34.9
		33.4
		34.1
	High	39.6
		45
		42.3
1949	Low	10.6
		14.1
		12.3
	High	21.1
		22.7
		21.9

the right, showing different degrees of divergence of the planes of the oral lobe and aboral arms. In general, this divergence is greater in plutei with large aboral-arm angles.

In Figures 48–58 the range of arm angles in high-temperature forms is represented. The figures are arranged in order of increase of aboral-arm angles from the smallest observed in all high-temperature lots, slightly over 20 angular degrees in Figure 48, to the largest, approximately 120 degrees, in Figure 58.

It is evident from comparison of Figures 48–50 with Figures 34–36, the smallest aboral-arm angles at low temperature, that, even though these angles do not differ greatly, the high-temperature plutei differ from the low-temperature forms in development of wider oral lobes and somewhat greater divergences of the oral arms. The apical or animal region, the high end of the polar gradient of the embryo from which the oral lobe develops, attains larger size at high temperature than at low. Extending this

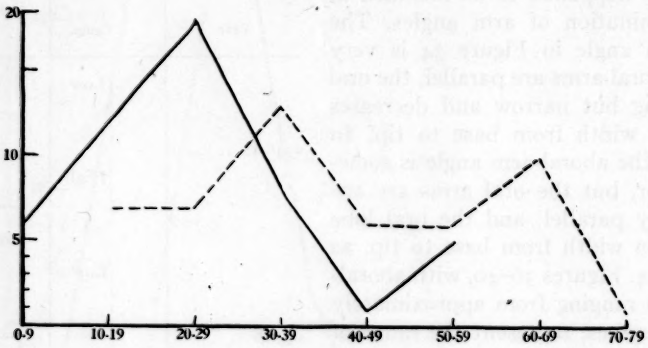


FIG. 30.—Frequencies of oral-arm angles of fully developed plutei in lots of 50 each at 12° C. (*continuous line*) and 22°–24° C. (*broken line*). From 1948 material. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

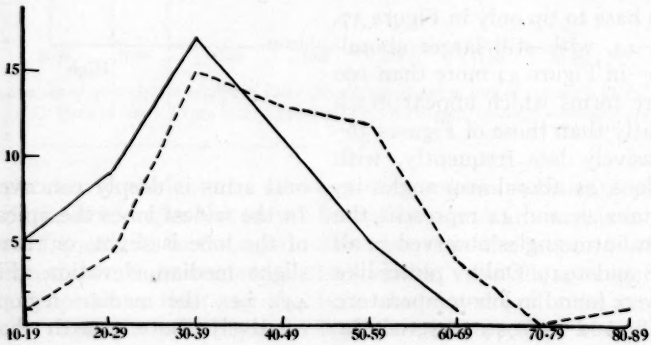


FIG. 31.—Frequencies of oral-arm angles of fully developed plutei in lots of 50 each at 12° C. (*continuous line*) and 22°–24° C. (*broken line*). From other containers of 1948 material than Fig. 30. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

comparison to the forms with larger angles—Figures 51–58 at high temperature and Figures 37–44 at low temperature—it is evident that the oral lobes are wider and the oral-arm angles larger in the high- than in the low-temperature forms. In these sample figures of forms from low and high temperatures, the attempt has been made to avoid selection for any particular feature other than aboral-arm angles. Moreover, on microscopic examination of material from the two temperatures, the differences in the oral lobes are evident at once. Microscopic examination of the cultures shows clearly what the graphs show somewhat more exactly, i.e., that plutei with the smaller aboral- and oral-arm angles are less numerous, those with larger angles more numerous, in the high- than in the low-temperature lots. Figures 51–55 represent approximately the range of the higher frequencies at high temperature. Forms like Figures 56–58 are less frequent but clearly more numerous in high than in low temperatures. No individuals with aboral-arm angles as large as in Figure 58 have been seen in any low-temperature lot.

The outlines of plutei in side view in Figures 59 and 60 are essentially similar to Figures 45–47 from low-temperature

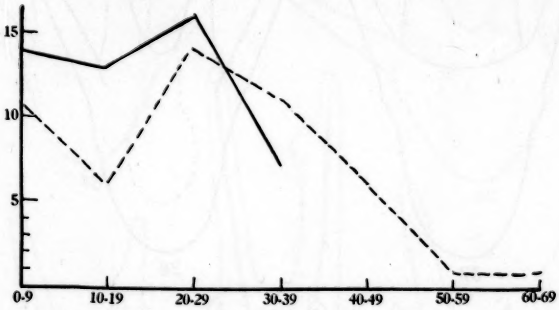


FIG. 32.—Frequencies of oral-arm angles of fully developed plutei in lots of 50 each at 12° C. (*continuous line*) and 20°–22° C. (*broken line*). From 1949 material. *Abscissae*, 10-degree classes; *ordinates*, numbers of individuals.

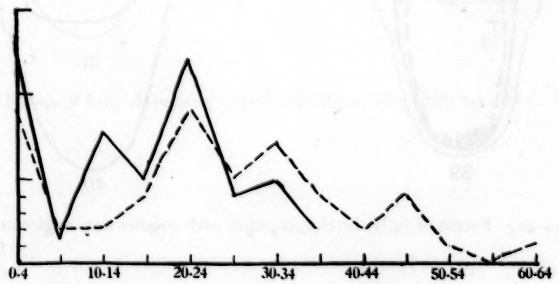
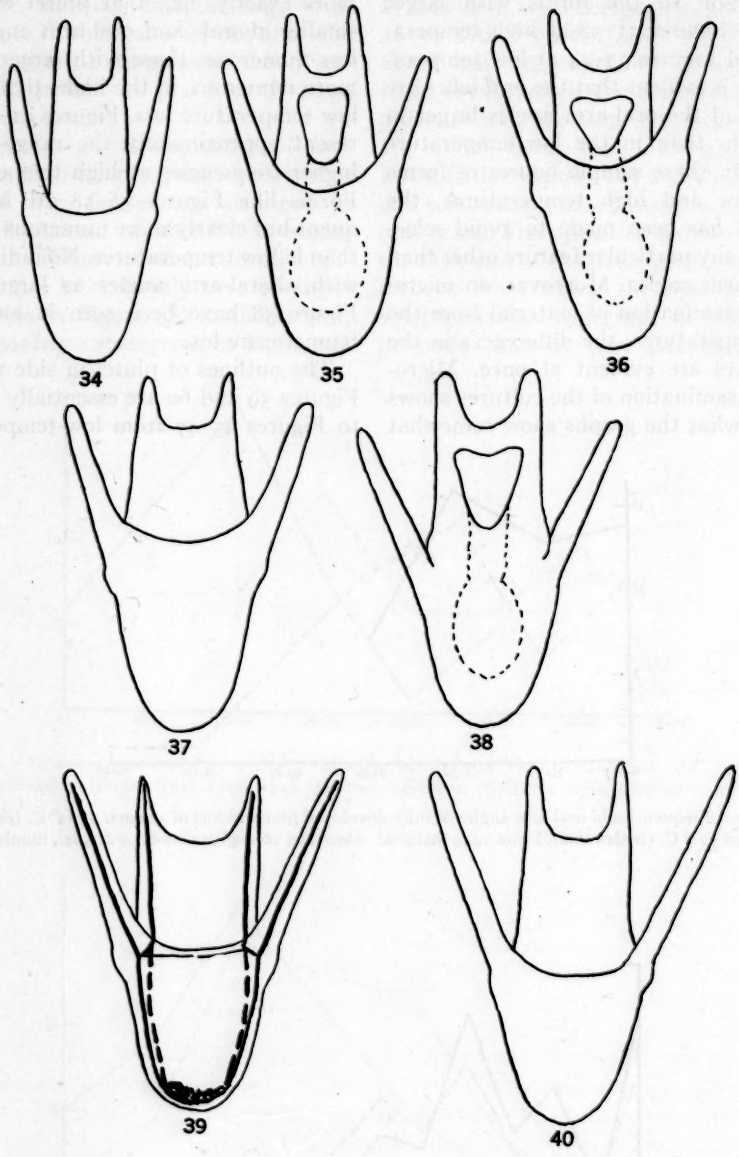
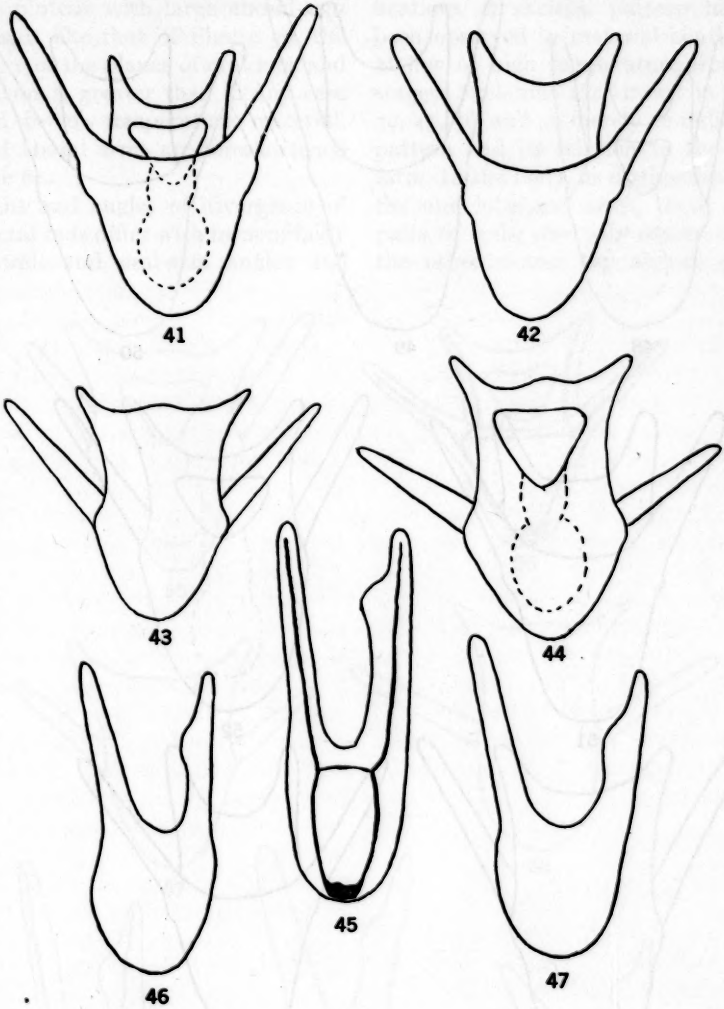


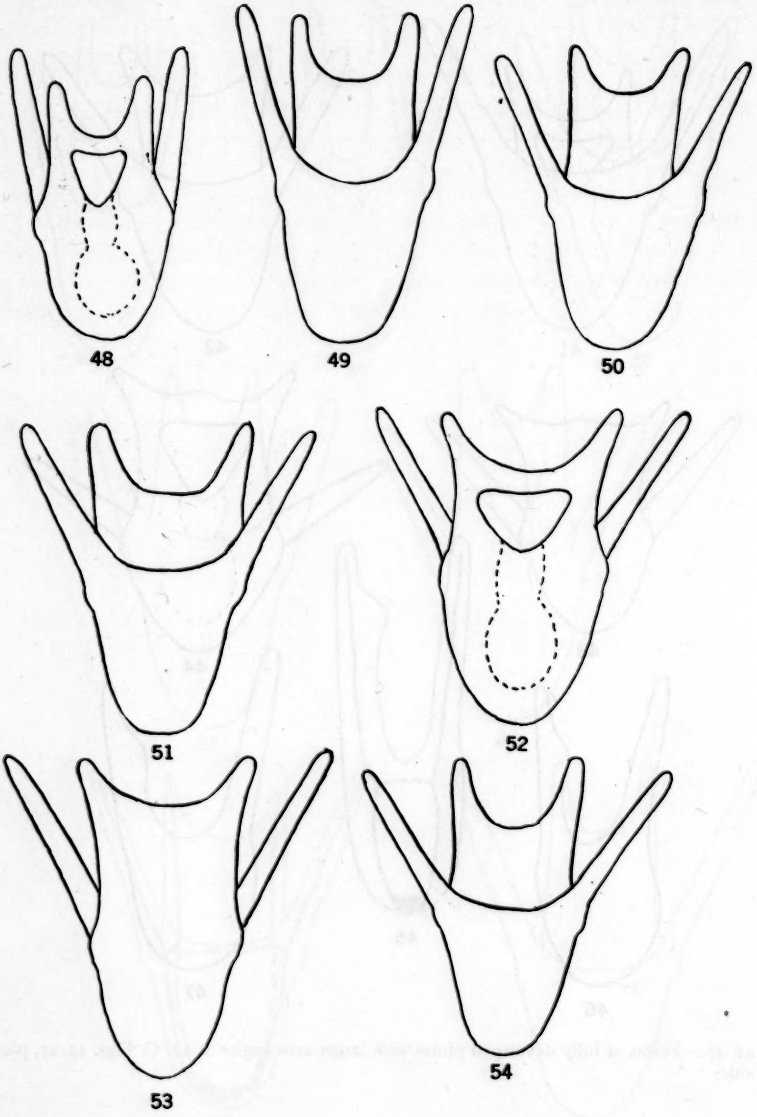
FIG. 33.—Frequencies of oral-arm angles from the same lots and plotted in the same manner as Fig. 32 but from 5-degree classes for comparison.



FIGS. 34–40.—Forms of fully developed plutei with smaller arm angles at 12° C.



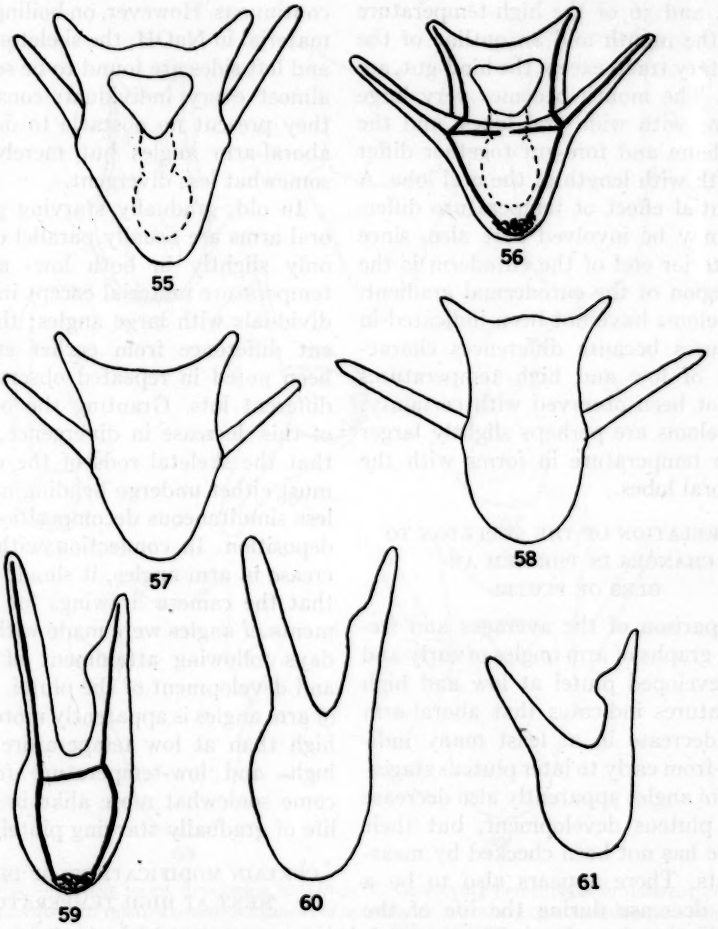
FIGS. 41–47.—Forms of fully developed plutei with larger arm angles at 12° C. Figs. 45–47, plutei seen from the side.



FIGS. 48–54.—Forms of fully developed plutei with smaller arm angles at 20°–22° C.

animals; but in Figure 61, a high-temperature pluteus with large aboral-arm angle about like that of Figure 56, the divergence of the planes of oral lobe and aboral arms is greater than in any case observed in low-temperature material. Oral and aboral arms are foreshortened in Figure 61.

Lengths and angles of divergence of the skeletal rods differ with form of body and aboral- and oral-arm angles and with width of oral lobe, but other modifications of skeletal pattern have not been observed in material continuously at low or high temperature from early stages. Skeletons are drawn in Figures 39, 45, 56, and 59 merely to indicate the pattern and its relation to the general form. In the body, as distinguished from the oral lobe and arms, there are two pairs of rods, one pair nearer the oral, the other nearer the aboral, side. In



FIGS. 55–61.—Forms of fully developed plutei with larger arm angles at 20°–22° C. Figs. 59–61, plutei seen from the side.

Figure 39 the rods near the aboral side are drawn in unbroken line; those near the oral side and lying deeper in this aboral view, in broken line. In Figure 45 half the skeleton is shown as seen from the side. In Figure 56 the skeleton of the oral lobe and aboral arms and the aboral part of the body skeleton are shown. Figure 59, like Figure 45, shows half the skeleton in side view.

In Figures 35, 36, 38, 41, and 44 of the low-temperature forms and Figures 48, 52, 55, and 56 of the high-temperature forms the mouth and an outline of the alimentary tract, except the hind-gut, are shown. The mouth becomes very large in forms with wide oral lobes, and the stomodeum and fore-gut together differ in length with length of the oral lobe. A differential effect of temperature differences may be involved here also, since the anterior end of the entoderm is the high region of the entodermal gradient. The coeloms have not been indicated in the figures because differences characteristic of low and high temperatures have not been observed with certainty. The coeloms are perhaps slightly larger at high temperature in forms with the larger oral lobes.

THE RELATION OF THE SKELETON TO CHANGES IN THE ARM ANGLES OF PLUTEI

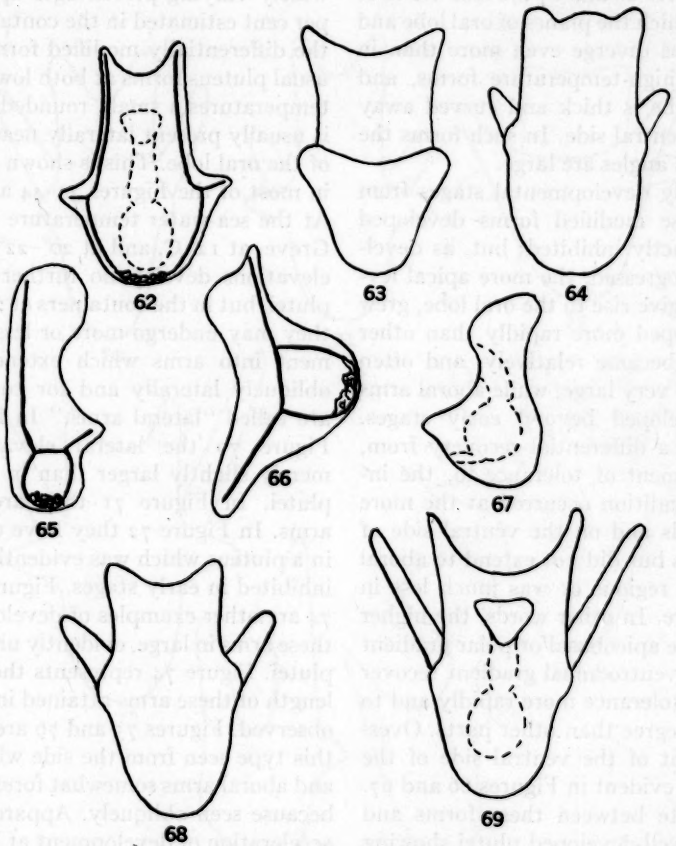
Comparison of the averages and frequency graphs of arm angles of early and fully developed plutei at low and high temperatures indicates that aboral-arm angles decrease in at least many individuals from early to later pluteus stages. Oral-arm angles apparently also decrease during pluteus development, but their decrease has not been checked by measurements. There appears also to be a further decrease during the life of the gradually starving plutei. The question of the relation of the skeleton to these changes is of interest. In early pluteus stages the skeletal structures of right and left sides of the individual are, of course, not connected. In the fully developed pluteus the longitudinal rods terminate dorsally in an irregular, fenestrated mass. In anesthetized animals and in formalin material the terminal structures of the two sides appear to be separate in some cases; in others they overlap, and it is often impossible to determine by observation whether they are separate or continuous. However, on boiling formalin material in NaOH, the skeletons of right and left sides are found to be separate in almost every individual; consequently, they present no obstacle to decrease of aboral-arm angles but merely become somewhat less divergent.

In old, gradually starving plutei the oral arms are usually parallel or diverge only slightly in both low- and high-temperature material except in some individuals with large angles; this apparent difference from earlier stages has been noted in repeated observations of different lots. Granting the occurrence of this decrease in divergence, it seems that the skeletal rods of the oral arms must either undergo bending or more or less simultaneous decomposition and redeposition. In connection with this decrease in arm angles, it should be noted that the camera drawings for measurements of angles were made within 1 or 2 days following attainment of full size and development of the plutei. Decrease of arm angles is apparently more rapid at high than at low temperature, so that high- and low-temperature forms become somewhat more alike in the later life of gradually starving plutei.

CERTAIN MODIFICATIONS OF DEVELOPMENT AT HIGH TEMPERATURE

It was noted in an earlier section (p. 143) that in most of the containers at the

highest temperature, 22°–25° C., and in two of those at 20°–22° C. differential modifications of development, not found in other containers, occurred in widely varying degree and percentage in different containers, in all cases together with large, well-developed plutei with high-temperature characteristics. Moreover, many of these plutei advanced further in development than is usual in unfed material. In all the containers in which the modifications beyond the usual range of pluteus form occurred, they were all of the same general type. Figures 62–67 are examples and indicate their relations to the gradient pattern. Figures 62–64 are outlines, with oral lobe above or below, of the more extreme modifications. Forms of this type range from a few individuals to at least 30–40 per cent estimated in different containers. They are characterized by relatively very large, usually both long and wide, oral lobes with various degrees of development of oral arms



FIGS. 62–69.—Pluteus forms developing at 22°–25° C. in varying percentages in different containers. Figs. 62–66, forms with relative overdevelopment of apical and ventral regions, resulting from inhibition in early stages, followed by differential recovery or perhaps differential tolerance in some cases. Figs. 65–67, modified forms seen from the side. Figs. 68 and 69, pluteus forms in the same containers as those in Figs. 62–67 but approaching the usual high-temperature proportions and arm angles.

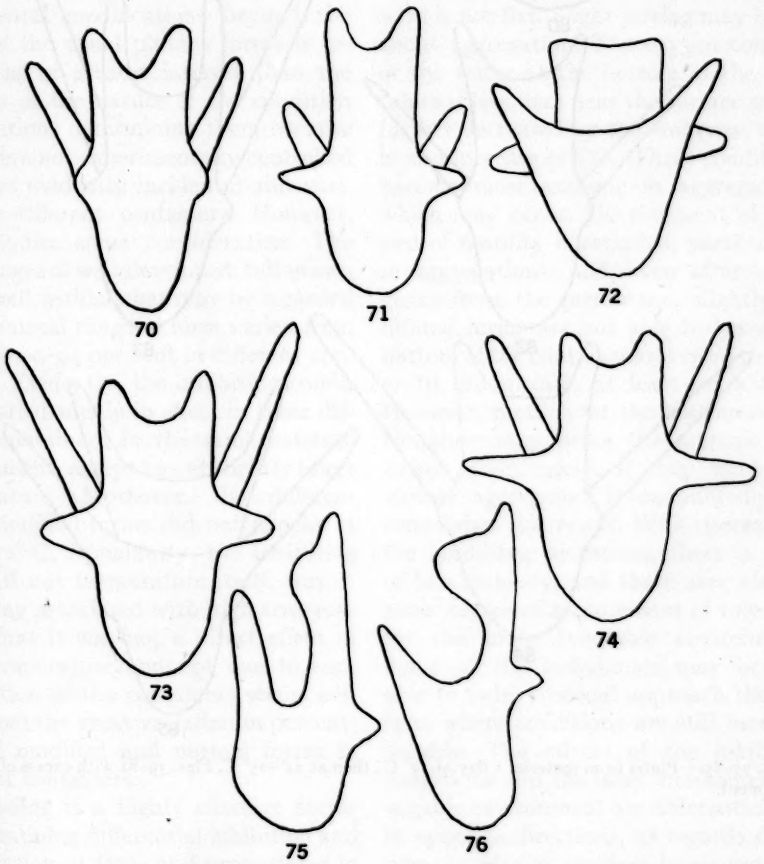
and greatly inhibited development of aboral arms; also the body is usually short (Figs. 62 and 63). Alimentary tract and skeleton are drawn only in Figure 62 but are essentially similar in all such forms. Figure 65 is a side view of a form essentially like Figure 62; it is largely oral lobe, and the thickening at the tip of the lobe is much larger than in the usual pluteus form. The skeleton corresponds in localization to the overdevelopment of the oral lobe and the inhibition of aboral arms. Figures 66 and 67 are side views of forms in which the planes of oral lobe and aboral arms diverge even more than in the usual high-temperature forms, and the oral lobe is thick and curved away from the ventral side. In such forms the aboral-arm angles are large.

The early developmental stages from which these modified forms developed were distinctly inhibited; but, as development progressed, the more apical levels, which give rise to the oral lobe, grew and developed more rapidly than other parts and became relatively, and often absolutely, very large, while aboral arms never developed beyond early stages. Evidently, a differential recovery from, or acquirement of tolerance to, the inhibiting condition occurred at the more apical levels and on the ventral side of these levels but did not extend to aboral and dorsal regions or was much less in degree there. In other words, the higher levels of the apicobasal or polar gradient and of the ventrodorsal gradient recover or acquire tolerance more rapidly and to a greater degree than other parts. Overdevelopment of the ventral side of the oral lobe is evident in Figures 66 and 67. Intermediate between these forms and the large, well-developed plutei showing the usual range of high-temperature forms are plutei with large oral lobes and short aboral arms (Fig. 68). These forms evidently represent lesser degrees of the same differential inhibition, followed by differential recovery or increased tolerance, as in Figures 62–64. A still closer approach to the usual high-temperature type is shown in Figure 69, a form with large oral lobe but medium aboral-arm angle. The frequencies of these forms are unquestionably higher in the containers with the modified forms just described than in high-temperature lots without such forms.

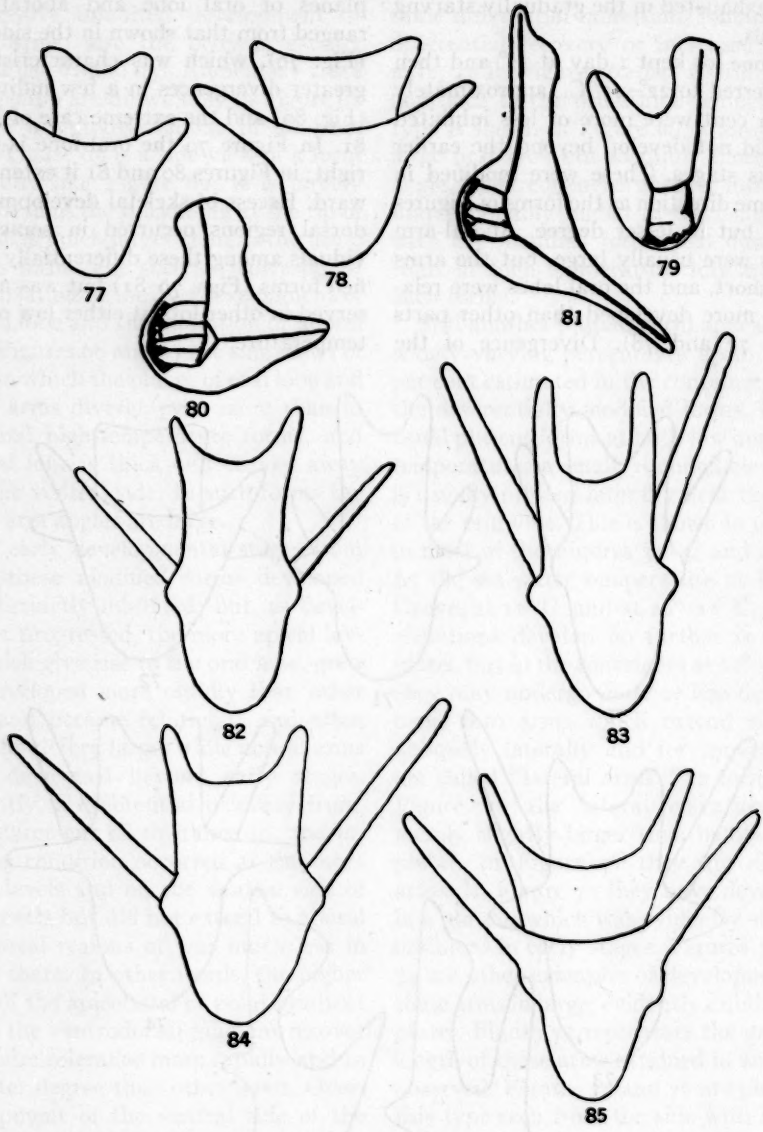
Still another pluteus form appeared in widely varying percentages up to 40–50 per cent estimated in the containers with the differentially modified forms. In the usual pluteus forms at both low and high temperatures a small, rounded elevation is usually present laterally near the base of the oral lobe. This is shown in outline in most of the Figures 34–44 and 48–58. At the sea-water temperature at Pacific Grove, at 12° C. and at 20°–22° C., these elevations develop no further in unfed plutei, but in the containers at 22°–25° C. they may undergo more or less development into arms which extend slightly obliquely laterally and for convenience are called "lateral arms." In forms like Figure 70 the lateral elevations are merely slightly larger than in the usual plutei. In Figure 71 they are distinct arms. In Figure 72 they have developed in a pluteus which was evidently slightly inhibited in early stages. Figures 73 and 74 are other examples of development of these arms in large, evidently uninhibited plutei. Figure 74 represents the greatest length of these arms attained in any case observed. Figures 75 and 76 are plutei of this type seen from the side with lateral and aboral arms somewhat foreshortened because seen obliquely. Apparently, the acceleration of development at high temperature in these individuals which have not been inhibited in early stages has permitted outgrowth of the lateral arms before material available for growth has

been exhausted in the gradually starvng animals.

In one lot kept 1 day at 12° and then transferred to 22°–25° C., approximately 10 per cent were more or less inhibited and did not develop beyond the earlier pluteus stages. These were modified in the same direction as the forms of Figures 62–69 but in lesser degree. Aboral-arm angles were usually large, but the arms were short, and the oral lobes were relatively more developed than other parts (Figs. 77 and 78). Divergence of the planes of oral lobe and aboral arms ranged from that shown in the side view (Fig. 79), which was characteristic, to greater divergences in a few individuals (Fig. 80) and the extreme case of Figure 81. In Figure 79 the oral lobe is at the right; in Figures 80 and 81 it extends upward. Excess of skeletal development in dorsal regions occurred in some individuals among these differentially modified forms (Figs. 79–81) but was not observed in other lots at either low or high temperature.



FIGS. 70–76.—Plutei with different degrees of development of a third pair of arms appearing in varying percentages at 22°–25° C. in the same containers as the forms of Figs. 62–69.



FIGS. 77–85.—Plutei from material 1 day at 12° C., then at 22°–25° C. Figs. 79–81 with excess of skeletal development.

Figures 82–85 represent the forms of fully developed plutei constituting approximately 90 per cent of this lot. In size and form they show, in general, high-temperature characteristics, extreme in Figure 85. Evidently 1 day at 12° C. did not prevent later development with these characteristics. However, outgrowth of lateral arms was not observed in any individual of this lot, but the rounded elevations from which these arms develop were slightly larger than in the usual unfed pluteus at lower temperatures.

The differential character of the developmental modifications beyond the range of the usual pluteus forms is regarded as of greater interest than the question of the nature of the condition or conditions determining them because these were not experimentally controlled and were evidently incidental and variable in different containers. However, they require some consideration. The percentages of well-developed, full-grown plutei well within what may be regarded as the normal range of form varied from 10–20 to 90–95 per cent in different containers. Evidently, the inhibiting conditions varied widely in effect in these different containers in the same external environment, except two at slightly lower temperature. Moreover, the differentially modified forms did not appear at all at 12° C. Apparently, the inhibiting factor, if not temperature itself, was in some way associated with high temperature. That it was not a direct effect of high temperature and not due to contamination of the containers seems evident from the great variation in percentages of modified and normal forms in different containers.

Crowding is a highly effective factor in determining differential inhibition and modification of form and proportions in echinoderm development, apparently by lack of oxygen or by accumulation of CO_2 or both. In distributing the material for the temperature experiments, it was believed that the numbers of embryos in a single container were not large enough to produce effects of crowding. However, in earlier experiments material had not been subjected to such high temperatures. The oxygen content of the water was, of course, lower at the high temperature, and in some containers there was more or less aggregation on the bottom. Even before hatching, the embryos may slide over the glass and aggregate in the lowest part if the bottom of the bowl is not flat. Slight jarring may bring about aggregation. The oxygen content of the water at the bottom of the container is less than near the surface and is further decreased by the embryos; there is also increase of CO_2. These conditions become most extreme in aggregations which may occur. Development of cilia and of motility is retarded, particularly in aggregations; and, even after emergence from the membrane, slightly inhibited forms are not able to leave the bottom of the container and swim toward or to the surface, at least for a time. However, motility at the bottom of the container may bring the animals into better environment; if they were previously aggregated, it undoubtedly accomplishes this result. With decrease of the inhibiting conditions, there is more or less recovery, and there may also be some degree of acquirement of tolerance for the more favorable environment. Some of the individuals may become able to swim free and approach the surface, where conditions are still more favorable. The effects of the inhibiting conditions and the later increasingly favorable environment are differential, but in opposite directions, as regards development. Higher gradient levels are most inhibited and, if the inhibition is not extreme, also recover or develop toler-

ance more rapidly and more completely than lower levels and become relatively larger and more differentiated than the lower, less active levels, as in Figures 62–67. The highly variable percentages of these secondarily modified forms in different containers is undoubtedly to be accounted for by differences in numbers and by absence, occurrence, and degree of aggregation. In the containers with smaller numbers or with little or no aggregation, many individuals were not appreciably inhibited and succeeded in reaching upper water levels early enough for development as large, fully differentiated plutei, often with a third pair of arms, i.e., with further progress toward metamorphosis than is usual in unfed plutei (Figs. 71–76). Between the extreme secondary modifications of Figures 62–67 and the large plutei with the third pair of arms there are intermediate forms of all degrees. In all the individuals in these containers there is differential acceleration of development and apparently of differentiation. In the secondarily modified forms the acceleration follows a primary inhibition; in the large, advanced plutei, development was accelerated from early stages without appreciable inhibition, but even in these the acceleration is differential, as the high frequency of large oral lobes and arm angles indicates. The intermediate forms result from different degrees of inhibition preceding the acceleration. The inhibition is regarded as due to incidental crowding at high temperature and is therefore only indirectly a temperature effect. The acceleration, on the other hand, is a direct effect of the high temperature. The secondary modifications of development in these high-temperature lots were all of the same general character, differing only in degree. These lots containing this wide range of forms are regarded as of special interest as examples of the range of local, experimentally uncontrolled conditions, affecting development differentially, which may occur in a single container at high temperature without agitation of the water except by the swimming larvae. It is hoped that further data along this line may be obtained.

DISCUSSION

The question whether the differences in averages and frequencies of arm angles of the plutei developing at low and high temperatures are real or merely results of a faulty method of sampling requires consideration. First of all, the measurements of arm angles confirm the differences directly visible on examination of low- and high-temperature lots of living plutei. Moreover, three temperature pairs from material, representing two breeding seasons of successive years, show essentially similar effects of temperature on the arm angles, as well as on forms and proportions of the plutei. No evidence has been obtained to indicate that plutei with either large or small arm angles or large or small oral lobes lie more frequently on the aboral arms or on the oral lobe than others do. Some plutei, usually those lying with oral lobe below, are tipped slightly to one side, so that the aboral arms appear not quite symmetrically, but the rounded form of the body dorsal to the arms and lobe determines this position. Arm angles were not drawn in these animals unless they were brought into symmetrical position. It is believed to be beyond question that the differences in arm angles at low and high temperatures are real and not due to errors of sampling. The higher frequencies of large, wide oral lobes at high temperature are evident on examination.

Development at 20°–22° C. requires approximately half as much time as development at 12° C. However, the point

of chief importance as regards the accelerating effect of the higher temperature is that it is differential rather than uniform and that the differential effect is directly related to the gradient pattern. Development is most accelerated at the more apical levels of the polar component of gradient pattern and at the ventral levels of the ventrodorsal component, with the result that the frequencies of relatively or absolutely large size of these regions are greater at high than at low temperature. The apical region is the high end of the polar component of the gradient pattern; the ventral region is the high end of the ventrodorsal component, as shown by intracellular oxidation and reduction of indicators and by susceptibility to various inhibiting agents. This differential acceleration at high temperature becomes evident in increased frequency of large, wide oral lobes, which develop from the high end of the polar gradient, and in larger angles of divergence of aboral and oral arms, i.e., in relative or absolute increase in size of the ventral region, the high region of the ventrodorsal gradient component. The higher gradient levels are more susceptible to temperature because they represent higher metabolic levels than other regions of the gradient pattern and react more rapidly and to a greater degree than lower levels. That very slight gradient differences are sufficient to determine differentials in development is suggested by the slight median elevation which often appears at the tips of the wide oral lobes at high temperature. This seems to indicate a slightly greater effect of high temperature in the median apical region than laterally.

If we regard the forms developing at 12° C. as normal, those developing at 20°–22° C. are differentially modified; if the latter are normal, the low-temperature forms are differentially modified. In other words, there is no normal pluteus form independent of external environment.

The secondarily modified forms like Figures 62–67 are of interest as cases of slight inhibition of early development regarded as due to various degrees of crowding at high temperature in different containers, followed by differential acceleration by the high temperature as the larvae became sufficiently active to escape from the inhibiting conditions at the bottoms of the containers. The differential acceleration in these forms shows the same relation to the gradient pattern as in the fully developed high-temperature plutei, except that it is limited almost entirely to the higher gradient levels. The lower gradient levels, reacting less rapidly, remain, to a large extent, inhibited.

The frequent development of a third pair of arms at the highest temperature used (Figs. 70–76) is of interest as showing that sufficient acceleration of development by high temperature may determine progress toward metamorphosis beyond that usually occurring in plutei without food.

The excess of skeletal development observed in many individuals subjected to 22°–25° C. after 1 day at 12° C. but not seen in lots kept continuously at the high temperature from early stages is apparently associated with the acceleration resulting from the change in conditions occurring at a certain stage of development. Mesenchyme cells are apparently relatively highly susceptible to external conditions, and excess of skeletal development has been observed frequently in recovery from moderate effects of inhibiting agents.

In general, the data of this paper constitute further evidence of the differential effects on morphogenesis of purely quantitative differences in external conditions

and of the definite relations of these effects to the physiological gradient pattern shown by various methods to be present.

SUMMARY

1. The fully developed pluteus stage of the sand dollar, *D. excentricus*, is attained in 96 hours or slightly longer time at 12° C. and in 48 hours at 20°–22° C., i.e., the temperature coefficient for 10° C. is approximately 2, perhaps slightly more.

2. The accelerating effect of the higher temperature is differential. Averages of angles of divergence and frequencies of large angles of aboral and oral arms and of large, wide oral lobes are greater at the high than at the low temperature.

3. Larger aboral-arm angles at the higher temperature are present at early, as well as later, stages of arm development. There is, in general, a decrease in aboral- and oral-arm angles from earlier to later pluteus stages and in at least many individuals a further decrease during the life of the gradually starving pluteus.

4. The differences in development at low and high temperatures are definitely related to the gradient pattern. Apical and ventral regions of the embryo and early larva are the regions of most rapid intracellular oxidation and reduction of indicators and of greatest susceptibility to various external inhibiting agents. The oral lobe develops from the apical region, and the ventral region extends from the tip of the oral lobe to the aboral arms. The larger oral lobes and arm angles developing at the higher temperature result from the greater susceptibility of the higher gradient levels to the accelerating effect of high temperature. They react more rapidly and to a greater degree than the lower levels.

5. In most of the lots subjected to temperatures of 22°–25° C., numbers of individuals were sufficient to determine slight inhibition of early development in widely varying percentages of the animals in different containers as an effect of crowding indirectly associated with the high temperature. These inhibitions were followed by partial differential recovery or perhaps also differential acquirement of tolerance in some individuals. The resulting secondary differential developmental modifications were all of the same general character and consisted of forms with relatively large oral lobes and ventral regions but little development of aboral arms. These forms represented differential acceleration of development at the higher gradient levels by the high temperature, with more or less inhibition still persisting at the lower, less rapidly reacting levels.

6. These secondary modifications show the same relations to the gradient pattern as do the fully developed high-temperature plutei; but the differential in morphogenetic effect is greater in consequence of the preceding developmental inhibition because the lower gradient levels react less rapidly and to a lesser degree after escape from the inhibiting conditions.

7. In the same containers with the inhibited and secondarily modified forms, fully developed plutei with the large aboral- and oral-arm angles characteristic of high temperature also appeared in numbers ranging from estimated percentages of 5–10 up to 50 or more. These evidently escaped the local crowding in early stages. Varying numbers of these up to at least half showed further progress toward metamorphosis than is usual in plutei without food, by development of a third pair of arms laterally.